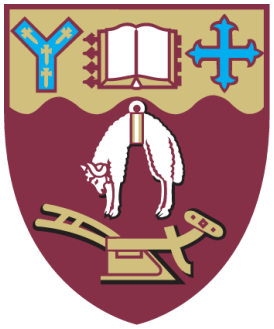
# The Characterisation of Putative Nuclear Pore-Anchoring Proteins in *Arabidopsis thaliana*



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# Contents

# List of Figures

# List of Tables

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# Abstract

The nuclear pore complex (NPC) is perhaps the largest protein complex in the eukaryotic cell, and controls the movement of molecules across the nuclear envelope. The NPC is composed of up to 30 proteins termed nucleoporins (Nups), each grouped in different sub-complexes. The transmembrane ring sub-complex is composed of Nups responsible for anchoring the NPC to the nuclear envelope. Bioinformatic analysis has traced all major sub-complexes of the NPC back to the last eukaryotic common ancestor, meaning that the nuclear pore structure and function is conserved amongst all eukaryotes. In this study *Arabidopsis* T-DNA knockout lines for these genes were investigated to characterise gene function. Differences in plant growth and development were observed for the *ndc1* knockout line compared to wild-type but *gp210* plants showed no phenotypic differences. The double knockout line *gp210 ndc1* was generated through crosses to observe plant response to the knockout of two anchoring-Nup genes. No synergistic affect from this double knockout was observed, suggesting that more, as yet unidentified Nups function the transmembrane ring in plants. The sensitivity to nuclear export inhibitor leptomycin B (LMB) was tested also for knockout lines, although growth sensitivity to the drug was not observed. Nucleocytoplasmic transport of knockout lines was measured in cells transformed by particle bombardment. To express fluorescent protein constructs actively transported through the NPC, localisation of protein determined the nucleocytoplasmic transport of the cell. The *ndc1*single knockout and the double knockout *gp210 ndc1* exhibited decreased nuclear export. Further experiments in determining NDC1 localisation and identification of other Nups in the transmembrane ring sub-complex would bring a more comprehensive understanding to the plant NPC.

# Abbreviations

| | |
|---|---|
| **-ve** | Negative control |
| **+ve** | Positive control |
| **µE.m$^{-2}$.s$^{-1}$** | Microeinstein per m$^{-2}$ s$^{-1}$ |
| **ANOVA** | Analysis of variance |
| **BLAST** | Basic local alignment search tool |
| **bp** | Base pairs |
| **DMSO** | Dimethyl sulfoxide |
| **EDTA** | Ethylenediaminetetraacetic acid |
| **Fsc** | Fasciated |
| **FG** | Phenylalanine-glycine |
| **GFP** | Green fluorescent protein |
| **HM** | Homozygote |
| **HMM** | Hidden markov models |
| **HT** | Heterozygote |
| **IP** | Insert primer |
| **KO** | Knockout |
| **LB** | Lysogeny broth |
| **LMB** | Leptomycin B |
| **LP** | Left border primer |

| | |
|---|---|
| **NES** | Nuclear export signal |
| **NPC** | Nuclear pore complex |
| **Nup** | Nuclear pore protein |
| **PCR** | Polymerase chain reaction |
| **psi** | Pounds per square inch |
| **PUM** | Pumilo homology domain protein |
| **RFP** | Red fluorescent protein |
| **RP** | Right border primer |
| **SEM** | Standard error of the mean |
| **TAIR** | The *Arabidopsis* information resource |
| **TE** | Tris and EDTA, components of TE buffer |
| **T-DNA** | Transfer DNA |
| **v/v** | volume/volume |
| **w/v** | weight/volume |
| **WT** | Wild-type |
| **YFP** | Yellow fluorescent protein |

# Glossary

| | |
|---|---|
| **Bolting** | The production of the flowering stem in a plant. |
| **Cross** | The deliberate interbreeding of plants with desirable characteristics to produce new lines with the desirable properties. |
| **Fasciation** | An unusual plant growth form in with the apical meristem becomes elongated perpendicularly to the direction of growth producing flattened band-shaped tissue. |
| **Forward Genetics** | Genetic analysis that proceeds from phenotype to genotype. |
| **Leptomycin B** | Potent nuclear export inhibiting drug first identified in *Streptomyces*. |
| **Microeinstein** | A measure of irradiance |
| **Nuclear Envelope** | A double lipid bilayer separating contents of the nucleus to the cytoplasm in eukaryotic cells. |
| **Nuclear Pore Complex** | Large protein complexes that are inserted in the nuclear envelope facilitating and regulating the exchange of materials between the nucleus and cytoplasm. |
| **Nucleocytoplasmic Transport** | The active transport of molecules through the nuclear pore complex. |
| **Opisthokonts** | A term for the broad group of eukaryotes which include the animal and fungal kingdoms. |
| **Pleiotropic** | The influence of one gene on multiple phenotypic traits. |
| **Reverse Genetics** | Genetic analysis that proceeds from genotype to phenotype. |

| | |
|---|---|
| **Self** | The deliberate self-fertilization of an individual plant that is self-compatible to produce progeny with desirable properties of the parent. |
| **Silique** | The seed capsule (fruit) of *Arabidopsis*. |
| **Synergistic** | Interaction of multiple genes enhancing the effect to an extent that cannot be produced singularly. |
| **Transmembrane ring** | A nuclear pore sub-complex responsible for attaching the nuclear pore complex to the nuclear envelope. |
| **T-DNA** | The transferred DNA of the tumor-inducing plasmid from the bacteria *Agrobacterium tumefaciens* used for insertional mutagenesis. |

# Chapter 1

# Introduction

## 1.1 Overview

The nuclear pore complex (NPC) is essential in the transport of cellular components between the nucleus and the cytoplasm as it is the only gateway between the two compartments. The composition of the nuclear pore complex in animals and fungi has been well characterised and it consists of many different protein subunits. These nuclear pore proteins are referred to as nucleoporins (Nups). By comparison, the characterization of plant nuclear pore complexes is poor, with many of the nuclear pore proteins undescribed. However, all major protein sub-complexes of the NPC have been traced back to the last eukaryotic common ancestor (Neumann et al. 2010), and many individual Nups can be identified in highly divergent species using sequence homology (Hetzer et al. 2005).

One sub-complex of the NPC thats has yet to be fully characterised in plants is the transmembrane ring, which comprise of anchoring-Nups. These are transmembrane proteins that span the lipid bilayer of the nuclear envelope and which are responsible for attaching the nuclear pore complex to the nuclear membrane. In other eukaryotes, three distinct membrane anchoring proteins are known to anchor the pore complex. These are NDC1, GP210, and POM121. Through bioinformatic analyses of the *Arabidopsis* genome, two genes homologous to NDC1 and GP210 have been identified (Neumann et al. 2010). These are At1g73240 and At5g40480. In characterising whether these homologs share function to their vertebrate and fungi counterparts, T-DNA insertion knockout plant lines will be studied.

## 1.2 The nuclear pore complex

Nuclear pore complexes are large protein complexes embedded in the nuclear envelope surrounding the nucleus. These serve as the interface between the cytoplasm and the nucleoplasm, and form the only gateway for the exchange of macromolecules between the two compartments (Tran and Wente 2006). Transported molecules include; RNA, proteins, and other molecules that support cellular function. Not only does the NPC serve as the gateway for the nucleus in eukaryotic cells but its components are also involved in regulatory processes of gene expression within the cell (D'Angelo and Hetzer 2008). The NPC plays an important role in plant development through the regulation of critical genes. Nups have shown to bind and regulate the activity of several developmental genes (D'Angelo et al. 2012; Kalverda et al. 2010).

The NPC possess 8-fold rotational symmetry consisting of specific sub-complexes; cytoplasmic fibrils, the central core, and the nuclear basket. NPC size varies among eukaryotes with plant and vertebrate NPCs larger than yeast. Using field emission scanning electron microscopy, NPC diameters of ~105 nm in tobacco, 110~120 nm in vertebrates, and ~95 nm in yeast were observed (Fiserova et al. 2009; Goldberg and Allen 1996; Kiseleva et al. 2004). The NPC contains a 9 nm aqueous nuclear pore through which molecules smaller than 30 kDa such as ions, small metabolites and small proteins can diffuse readily across (Gasiorowski and Dean 2003). Molecules possessing a mass >40 kDa need to be actively transported through the NPC. Nucleocytoplasmic transport is a complex process that is carried out by a large family of transport receptor proteins known as karyopherins, these include importins and exportins depending of the direction of transport (Mosammaparast and Pemberton 2004). These karyopherins are regulated by the Ran-GTP system (Azuma and Dasso 2000).

The NPC is a protein complex that consists of about 30 distinct Nups within the 120 MDa supramolecular complex (Rout and Aitchison 2000). These Nups are grouped into three functional classes (Figure 1.1).

i) Structural Nups stabilise the nuclear membrane curvature at nuclear pores and provide scaffolding in assembling other peripheral Nups.
ii) Phenylalanine-glycine (FG) Nups contribute to the permeability barrier for nonspecific transport and have direct binding sites for transport receptors that

facilitate nucleocytoplasmic movement. At least a third of the NPC is comprised of Nups with a significant phenylalanine-glycine (FG) repeat domain which directly binds to transport receptors and mediates active transport through the NPC (Ryan and Wente 2000).

iii) Transmembrane ring Nups anchor NPCs to the nuclear envelope (Adams and Wente 2013; Terry and Wente 2009).

Studies have shown conservation of the NPC across eukaryotes with all major protein sub-complexes in the NPC traceable to the last eukaryotic common ancestor (Neumann et al. 2010).

Since the NPC oversees the trafficking in and out of the nucleus, it plays a key function in plant development and effects a myriad of plant processes and interactions including hormone and stress responses, pathogenic responses and flowering control (Meier and Brkljacic 2009). Not only are NPCs the gates for trafficking molecules in and out of the nucleus but they are also involved in the regulatory processes on both sides of the nuclear envelope (Strambio-De-Castillia et al. 2010). The NPC associates with molecules and structures in the cytoplasm and nucleoplasm through its cytoplasmic filaments and nuclear basket. This enables regulatory processes such as transcriptional regulation in the nucleus and protein synthesis regulation in the cytoplasm. Characterising the dynamics in structure and regulation of plant NPCs would assist in understanding nucleocytoplasmic transport and NPC associated regulation.

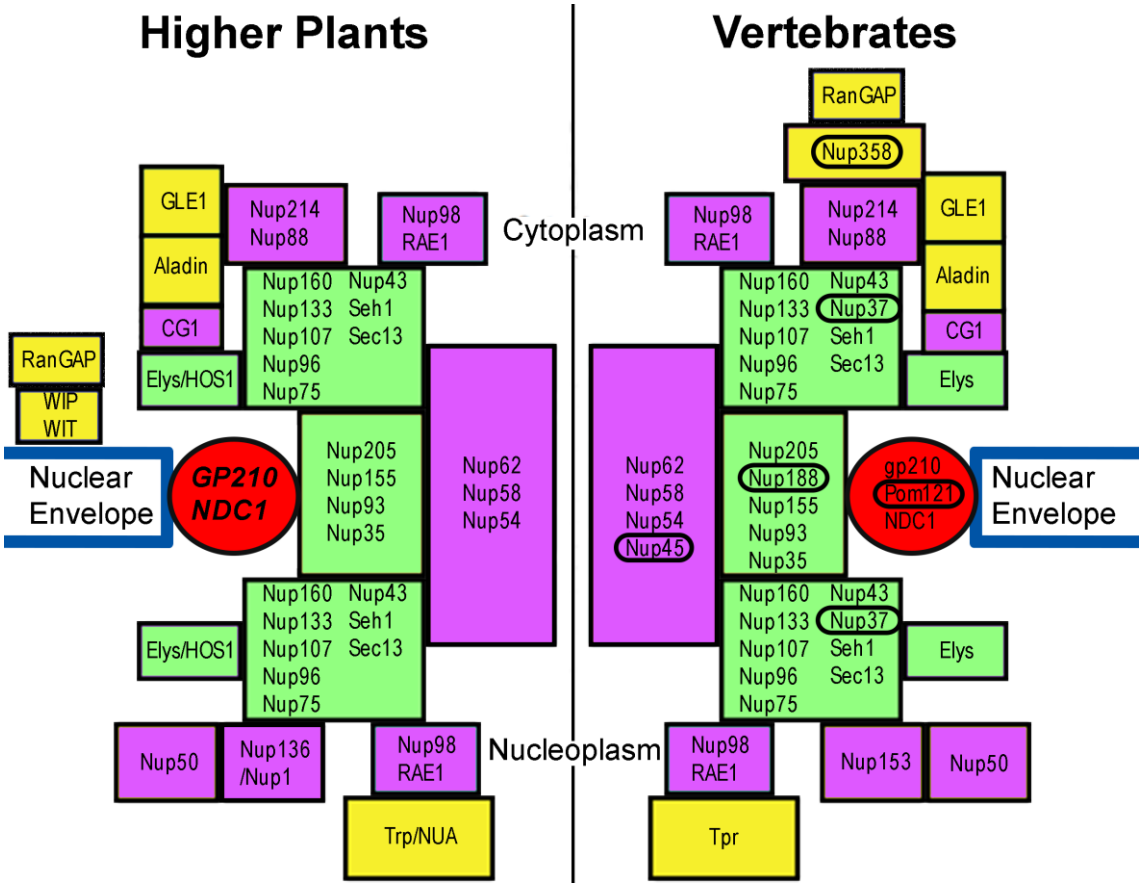


**Figure 1.1.** Characterised Nups in the nuclear pore complex of higher plants compared to Nups found in vertebrates. Proteins in bold are the putative plant Nups investigated in this study. Proteins circled are vertebrate Nups that have no identified plant equivalent. Different colours indicate different functional classes of Nups (green: structural, purple: FG, red: transmembrane, yellow: other). Modified from Tamura et al. (2010).

## 1.3 Transmembrane nuclear pore anchoring proteins

All Nups are soluble except for transmembrane ring Nups (Mansfeld et al. 2006). The integration of the NPC to the nuclear envelope requires the fusion of the inner nuclear membrane and the outer nuclear membrane. It remains unclear how transmembrane Nups bridge this gap but it is believed luminal loops and domains of the proteins are used as docking sites (Stavru et al. 2006a). The linkage between soluble and transmembrane Nups is presently unclear. During mitosis in vertebrates and plants, the NPCs and nuclear envelope disassembles. Transmembrane Nups function as early intermediates in initiating NPC reformation and stabilisation following mitosis (Rout and Aitchison 2001).

It was initially thought that the nuclear pore anchoring system evolved either by convergence or was only restricted to opisthokonts (Bapteste et al. 2005). Later, it was thought that parts of the anchoring system evolved before the split of vertebrates and fungi (Bapteste et al. 2005).

Recent genomic analysis has however, confirmed that the transmembrane anchoring system was present in the last common ancestor of eukaryotes (Neumann et al. 2010). To date, three distinct transmembrane anchoring-Nups have been described in vertebrates. These are Pom121, NDC1, and GP210 (Mansfeld et al. 2006; Stavru et al. 2006a; Stavru et al. 2006b). Three transmembrane anchoring-Nups have also been described in fungi, Pom152p, Pom24p, and Ndc1p (Rout et al. 2000). Nup POM121 is detectable only in vertebrates and GP210 appears to have been secondarily lost in fungi (Hetzer et al. 2005; Mans et al. 2004). Pom152p and Pom24p are restricted to fungi, whereas NDC1/Ndc1p is universally conserved across eukaryotes and is also a component of the spindle pole body in fungi (Hetzer et al. 2005; Winey et al. 1993).

NPC transmembrane components have demonstrated importance in cellular processes with NDC1 shown to be essential in the viability of yeast (Winey et al. 1993), and in selective nuclear protein import in vertebrates (Yamazumi et al. 2009). Vertebrate NDC1 and Pom121 play important roles in post-mitotic NPC assembly (Antonin et al. 2005; Mansfeld et al. 2006). The loss of interaction between NDC1 anchoring the Nup ALADIN to the nuclear envelope has also been implicated in the pathogenesis of the human inheritable disease triple A syndrome (Kind et al. 2009). The transmembrane Nup GP210 was also found to be a regulator for genes essential to cell differentiation in vertebrates (D'Angelo et al. 2012).

While in plant Nups have not been described in such detail, proteins with convincing similarity to NDC1 and GP210 have been identified (Zhao et al. 2008). Nuclear pore anchoring proteins were identified in plants using HMM and BLAST bioinformatic analyses, it has been confirmed that the last common eukaryotic ancestor possessed a conserved anchoring system, and that anchoring-Nups NDC1 and GP210 can be identified in the Plantae kingdom (Neumann et al. 2006; Neumann et al. 2010). Putative transmembrane anchoring-Nups NDC1 and GP210 are embedded in the nuclear envelope anchoring the NPC (Figure 1.1). Moreover, a high level of sequence similarity was identified between *Arabidopsis* and vertebrate Nups that was not found in yeast. This suggests that the plant NPC has a higher similarity to the vertebrate NPC than to yeast (Tamura et al. 2010).

In *Arabidopsis*, the genes At1g73240 and At5g40480 were identified putative as putative homologs for NDC1 and GP210 respectively (Neumann et al. 2010). These genes have been annotated as protein coding genes and their expression is ubiquitous in the plant and throughout growth and development stages. At5g40480 has also been annotated as having an embryo defective phenotype in knockout lines (http://arabidopsis.org/servlets/TairObject?id=132136&type=locus, http://arabidopsis.org/servlets/TairObject?id=136600&type=locus, accessed June 19th 2013). A GFP fluorescent fusion construct was created for the *Arabidopsis* GP210 homolog At5g40480 and then stably expressed, this protein localised to the nuclear envelope (Tamura et al. 2010), similar experiments have yet to be conducted for *NDC1*/At1g73240. No plants homologs were identified for Nup POM121 (Neumann et al. 2010).

Transmembrane anchoring-Nups could provide a link between the central NPC framework and the nuclear membrane by interacting with soluble Nups or Nup sub-complexes (Mansfeld et al. 2006). Describing these Nups in plants would help find their roles and importance to the NPC and in plant development. Comparisons to their vertebrate and fungi counterparts could also be made furthering understanding of the evolution of the NPC.

## 1.4 T-DNA insertional gene knockouts in *Arabidopsis thaliana*

The most straightforward approach in studying gene function is to characterise the phenotypic changes associated with its total inactivation in a model plant (Bouché and Bouchez 2001). By far the most comprehensively studied flowering plant is *Arabidopsis thaliana*, the growth

of *Arabidopsis* research over the last 30 years has been exponential and has transformed the study of higher plants (Koornneef and Meinke 2010).

There are several features that contribute to *Arabidopsis* being the model organism in classical experimental genetics these include its small, extensively studied genome, small plant size meaning limited growth facilities are required, rapid regeneration time (5-6 weeks under optimum conditions), the ability to grow well under controlled conditions, a high fecundity (up to 10,000 seeds per plant), and the ease with which a mutant line can be maintained by self-fertilisation and outcrossed (Koornneef and Meinke 2010; Page and Grossniklaus 2002). *Arabidopsis* is also becoming a model system in quantitative genetics investigating population structure, plasticity, and evolution, this is because it allows that extension of genetic analyses to the molecular level (Mitchell-Olds 1995).

In studying gene function in *Arabidopsis*, insertional mutagenesis is a key tool. The two major advantages in insertional mutagenesis are that the mutants are labelled by the insert fragment of known sequence and an insertion within the coding region has a high probability of eliminating gene function (Parinov and Sundaresan 2000). T-DNA or transposons are usually employed as insertional mutagens in *Arabidopsis.* For studying gene function in *Arabidopsis*, there is an extensive and well-catalogued library of over 150,000 T-DNA insertional mutants created from pooled collections produced by several laboratories (O'Malley et al. 2007; Sussman et al. 2000). The *Arabidopsis* Information Resource (TAIR) is the main resource in *Arabidopsis* research providing a centralised, curated gateway to *Arabidopsis* biology, research materials, seed and DNA stock information with an online ordering system (Rhee et al. 2003).

Insertion mutants have been considered the best choice for investigating gene function because of the ability to screen lines either for reporter genes contained within the T-DNA insert or by probing each line by PCR with a gene and T-DNA specific primer pair (Krysan et al. 1999; McKinney et al. 1995). These mutants greatly accelerate gene function studies which enable researchers to directly order seed lines from the library of mutants with a T-DNA insert of their gene of interest (Alonso et al. 2003).

T-DNA insertional mutants have been used in many studies investigating plant Nups, with many Nup mutants exhibiting pleiotropic developmental defects which include smaller plant size, abnormal leaf arrangement, terminal floral structures and reduced fertility (Xu and Meier

2008). Insert mutants of genes homologous to transmembrane anchoring-Nups may produce similar phenotypes in plant growth due to the potential disruption of the NPC.

## 1.5 Hypothesis and objectives

The aim of this study is to characterize the function of *Arabidopsis thaliana* genes At1g73240 and At5g40480 that have been suggested to be homologous to transmembrane Nups GP210 and NDC1 (Neumann et al. 2010), and to be responsible for anchoring the NPC to the nuclear envelope. PCR will be used to isolate and confirm *Arabidopsis thaliana* lines containing homozygous T-DNA insertions. Then sequencing of the PCR products from screening will confirm the site of the T-DNA insertion within the open reading frame of these genes.

The growth of these knockout lines will be investigated to observe any changes in plant development occurring from the disruption of the anchoring-Nup homologs. The phenotypes of these plants will be characterised in determining how the knockout of these genes affects plant growth and their importance in overall plant development. To investigate a possible synergistic relationship between the two genes the knockout lines will be crossed together and the progeny containing both T-DNA inserts will be studied for any enhanced phenotypic response. Crosses will also be performed between knockout lines and a nuclear-GFP expressing line to help visualise the nuclear morphology and determine these gene homologs have a role in the nuclear structure.

The effects of a known nuclear transport-inhibiting drug, leptomycin B (LMB), on knockout lines will be determined, along with the effects of nuclear transport assayed using transient expression of a fluorescently-tagged protein pumilo homology domain protein (PUM) known to be actively transported through the NPC. We hypothesise that if the NPC is disrupted there would be an observable difference in plant growth under LMB and a difference in nucleocytoplasmic transport of knockout lines compared to wild-type through the use of the PUM construct.

# Chapter 2

# General Materials and Methods

## 2.1 *Arabidopsis* Growth Conditions

### 2.1.1 *Arabidopsis* seed planting

*Arabidopsis thaliana* seeds were surfaced sterilised in an Eppendorf tube for 90 s with a solution containing 50% (v/v) ethanol and 3% (v/v) hydrogen peroxide, and then extensively washed afterwards in sterile distilled water. Under sterile conditions, individual seeds were plated with about 5 mm spacing on 1.2% (w/v) agar (Bacto-agar, Difco Laboratories, Franklin Lakes, New Jersey, USA) plates containing Hoaglands solution, modified by the addition of 3% sucrose (w/v). Hoaglands solution contained: 2 mM $KNO_3$, 5 mM $Ca(NO_3)_2$, 2 mM $MgSO_4$, 1 mM $KH_2PO_4$, 90 µM iron-EDTA complex, 46 µM $H_3BO_3$, 9.1 µM $MnC1_2$, 0.77 µM $ZnSO_4$, 0.32 µM $CuSO_4$, and 0.11 µM $NaMoO_3$ (modified from Baskin et al. 1992). *Arabidopsis* plant lines used in this study are listed and described in Table 2.1.

### 2.1.2 Plant growth conditions

Planted seed plates were wrapped with parafilm and stored at 4°C for 2 d to stratify the seeds and synchronise their germination. The plates were placed vertically in a plant growth cabinet and grown at 21°C with 24 h light. The light intensity was 100 $\mu E.m^{-2}.s^{-1}$.

After 5 days, and once the seeds had germinated and the seedlings were of adequate size, they were transferred to pots (90 mm square, 1-2 seedlings per pot) that contained potting mix (Black Magic seed raising mix, Yates, Auckland, New Zealand). These pots were placed in a larger, clear plastic container (25.4 cm square) which was then covered in plastic wrap to ensure high humidity. After a week the plastic wrap was removed. The plants were grown at 21°C in either of two growth rooms with differing light cycles. Room 1 had a 10 h light / 14 h

dark cycle while room 2 had a 24 h light regime. Vegetative growth was favoured by the light/dark regime whereas early flowering was promoted by continuous light (Koornneef et al. 1991; Niwa et al. 2009).

*Arabidopsis* is a self-pollinating species. Plants were covered to prevent outcrossing, to promote the self-pollination for lines with a desired genotype, and also to comply with regulatory requirements for the containment of pollen and seeds. For covering plants, tubes were made from rolls of overhead transparency film (sheets of cellulose acetate). These tubes were wrapped around individual plants in pots as they bolted, and were about 2.5 cm in diameter and 20 to 30 cm high. As the tubes were open at the top to allow for air flow, all inflorescence stems were pruned to keep them within the cover to retain seeds and pollen.

### 2.1.3 Harvesting seed

Once plants had developed siliques, watering was stopped. This allowed the plants to dry out and the siliques to turn a golden-brown colour. At harvest, the transparency covers were removed and the inflorescences were cut off at the base. The plant material was placed in a large brown envelope to dry out completely. After one week, the seeds were sieved (250 mm diameter metal sieve, 400 µm pore size) from the plant material and stored at 4°C.

### 2.1.4 Crossing *Arabidopsis* lines

*Arabidopsis* lines were crossed to create progeny with T-DNA inserts and GFP expressing lines that would help visualise the cell nucleus and nuclear envelope. Crosses were also performed between different knockout lines to analyse the phenotype of double knockouts, as *Arabidopsis* lines with double T-DNA knockouts have been used to identify whether genes in the same family have different or redundant functions (Su and Li 2008).

*Arabidopsis* plants were crossed with a dissecting microscope using forceps and scissors. The dissecting microscope stage, tools and hands were sterilised with 95% (v/v) ethanol and air dried for 10 - 15 min to remove contaminating pollen, both before and between handling parent plants.

The pollen donor plant for the cross was chosen by having petals perpendicular to the flower body. By this stage the flowers should have had anthers releasing pollen. Under the dissecting microscope, several anthers were removed from their flowers and the release of pollen grains was confirmed. The female parent plant that had several young flower buds located near the

top of the inflorescence was pinned down to the dissecting stage using blu-tack. Other flower buds and flowers from the inflorescence were removed. The sepals and petals of the female parent plant were gently opened with forceps, and the stamens were removed. These were a green / yellow colour and were checked to ensure that they were free of pollen grains. Were any pollen to be present, another flower was used. The stigma was then pollinated with the male anthers. Once pollination was completed, petals and sepals were gently closed and a paper cover was put on the pistil to protect it from other pollen sources.

Following successful pollination, the pistil elongated as the seeds developed. Once the silique had fully elongated and dried to a golden-brown colour, it was removed from the plant. The siliques were dried in a small brown envelope for a week before being sieved. The seeds were then stored in 4°C for several days to increase frequency of germination before planting. Successful crossed plant lines are listed in Table 2.1.

Where possible, crosses were organised such that the male line (pollen donor) was genetically modified to homozygously-express GFP fusion proteins. This meant that germinated seed could be screened for the expression of GFP which would confirm that a successful cross had been performed.

## 2.2 Molecular biology

### 2.2.1 DNA extraction

DNA extraction and purification was conducted for the screening and sequencing of wild-type and T-DNA knockout plants. Plants chosen for DNA extraction had one or two leaves (100 mg of plant tissue or less) that were clipped from selected plants into an Eppendorf tube. A micropestle containing the abrasive powder celite (World Minerals, Auckland, New Zealand) was used to grind to the tissue at room temperature; buffer (from DNA purification kit) was then added immediately to the tissue after grinding.

A GenCatch Plant Genomic DNA Purification Kit (Epoch Life Sciences, Missouri City, Texas, USA) was used following the manufacturer's instructions to purify genomic DNA resulting in 200 µl of purified plant DNA in TE buffer (10 mM Tris, 1 mM EDTA). The DNA samples were then stored at -20°C.

### 2.2.2 Spectrophotometry

A NanoDrop spectrophotometer (Thermo Fisher Scientific, North Shore City, New Zealand) was used to measure the quantity and purity of DNA samples.

The NanoDrop utilises the absorbance peak of DNA (260 nm) and assesses the 260/280 nm and 260/230 nm ratios to calculate concentrations (ng/µl) and estimate the purity of samples respectively. Samples generally accepted as pure have 260/280 ratios between 1.8 - 2.0 (NanoDrop 2008).The values for concentration and purity of samples were given by the NanoDrop program.

### 2.2.3 PCR primer design

Primers for PCR screening of T-DNA lines were designed using T-DNA Express (powered by GEBD) on the Salk Institute Genomic Analysis Laboratory (SIGnAL) website (http://signal.salk.edu/tdnaprimers.2.html). Primers were also designed for within T-DNA inserts to determine their presence in particular lines, using DNAMAN (Lynnon Biosoft, Pointe-Claire, Quebec, Canada). Primers were designed to minimise potential primer dimers, hairpin structures and false priming. Primer lengths were designed with lengths between 18 and 25 nucleotides and with melting temperatures (Tm) between 55°C and 65°C. Table 2.2 lists primers for screening knockout lines and inserts.

**Table 2.1.** Plant lines used in the experiments.

| Code | Description | Stock Name | Insert Site | Vector | Insertion Line |
|---|---|---|---|---|---|
| WT | Wild-type Columbia | | | | |
| T11 | Nuclear GFP | CS84731 | | pEGAD | |
| T65 | At1g73240 KO, *ndc1* | CS829119 | Exon | pDAP101 | Syngenta (SAIL) |
| T74 | At5g40480 KO, *gp210* | SALK_043316 | Exon | pROK2 | SALK Confirmed T-DNA Project (SALK) |
| T79 | At1g73240 KO, *ndc1* | SALK_073352 | Promoter (At1g73250 exon) | pROK2 | SALK Confirmed T-DNA Project (SALK) |
| T83 | At5g40480 KO, *gp210* | SALK_138935C | Exon | pROK2 | SALK Confirmed T-DNA Project (SALK) |
| FT65 | T65 plant exhibiting fasciation | | | | |
| FT74 | T74 plant exhibiting fasciation | | | | |
| FT79 | T79 plant exhibiting fasciation | | | | |
| FT83 | T83 plant exhibiting fasciation | | | | |
| X1 | Double heterozygous nuclear-GFP *gp210*, T11 – T83 F1 cross progeny | | | | |
| X2 | Double homozygous nuclear-GFP *gp210*, X1 selfed progeny | | | | |
| X3 | Double heterozygous *ndc1 gp210*, X2 – T65 F1 cross progeny | | | | |
| X12 | Double homozygous *ndc1 gp210*, X 3 – selfed progeny | | | | |

**Table 2.2.** PCR primer sequences and PCR product fragment lengths.

| Code | Description | Forward Primer | Reverse Primer | Wild-type fragment (bp) | Knockout fragment (bp) |
|---|---|---|---|---|---|
| SAIL | PDAP101 T-DNA vector (For line T65) | | GCC TTT TCA GAA ATG GAT AAA TAG CCT TGC TTC C | | |
| SALK | pROK2 T-DNA vector (For lines T83, T79, T79) | | ATT TTG CCG ATT TCG GAA C | | |
| T65 | Insert into At1g73240 exon | ATA CAG ACA CAA GCC CAC CTG | TTT GGA TCT CCG AGT ATG GTC | 1081 | 642 |
| T83 | Insert into At5g40480 exon | TGA TGA TGT GTC TTT TGT GGC | CTC CCC GTC TTC CAC TTT ATC | 1232 | 747 |
| T74 | Insert into At5g40480 exon | CTT TTA CCT GTT TTG CAT GCC | TTT CAG TAG GGC CAT AGC ATG | 1152 | 540 - 840 |
| T79 | Insert into At1g73240 promoter located in At1g73250 exon | CTC AGC CAA TTC CTG AGT CTG | TTG CCA GAT AAG AAA AGC TGC | 1197 | 599 - 899 |
| RF | Screening for the phytopathogen *R. fascians* | CAC AGA CGC AAG CAA GGT TT | GGT TGA GCC CTT CCA CTT TC | 210 | |
| SAIL-1351 | SAIL insertion screening A | GTC CTA CAC GCC GAA ATA AAC | AGG GAA GAA AGC GAA AGG AGC | 1351 | |
| SAIL-914 | SAIL insertion screening B | CTG GAA CAA CAC TCA ACC CTA | GCA ACT TTA TCC GCC TCC ATC | 914 | |
| SALK-1452 | SALK insertion screening A | CAC CAG TAG CAC CAT TAC CAT | CTC CTG TCA TCT CAC CTT GCT | 1452 | |
| SALK-600 | SALK insertion screening B | CGG CGA GTT CTG TTA GGT CC | CCC AAT ACG CAA ACC GCC TC | 600 | |

### 2.2.4 PCR

PCR reactions were required for the amplification of DNA between genomic and insert primers to screen for wild-type and knockout plants. KAPA Taq 2X ready mix (KAPA Biosystems, Boston, Massachusetts, USA) was used, and reactions were conducted following the manufacturer's instructions. A master mix without the template and/or primers was prepared for multiple samples.

A standard 20 µl PCR reaction consisted of 10 µl Taq ready mix, 1 µl for each right and left genomic primer, 1 µl of insert primer, 5 µl distilled water, and 2µl of purified plant DNA. Primer concentrations were made to 10 µM. The tubes were spun beforehand and the reactions were carried out in a Multigene PCR machine (Labnet International, Woodbridge, New Jersey, USA). The PCR conditions were 95°C for 30 seconds (denaturation), 55°C for 30 seconds (annealing), 72°C for 90 seconds (elongation). These steps were run for 30 cycles before coming to a final hold of 4 °C.

### 2.2.5 Agarose gel electrophoresis

DNA agarose-gels were used throughout the project to quantify PCR products for sequencing and screening between wild-type lines and knockout lines containing the T-DNA insert. The different PCR product band lengths were separated by electrophoresis and would correspond to the predicted band length between the genomic primers or the insert primer with the right genomic primer.

Both 1% and 1.5% (w/v) agarose gels were used depending on optimal resolution for linear DNA length. Gels were prepared by dissolving agarose gel powder in 1x TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) and adding 2 µl of SYBR Safe DNA gel stain (Life Technologies, Carlsbad, California, USA) per 30 ml of agarose. Once the solution had cooled to around 60°C, the agarose was poured into a cradle containing the desired number of combs.

The gels were run between 70 V and 100 V on a BIO-RAD Power Pac 300 (Bio-Rad, Hercules, California, USA). The run would last for the appropriate time for the DNA to run the length of the gel or until bands were sufficiently separated and distinguishable. Since KAPA Taq 2X ready mix contained tracking dye, the PCR products did not require loading buffer to be added. However, when purified DNA or purified PCR products were run samples were loaded with Bioline 5x DNA loading buffer blue. For quantifying the size of the bands

5 µl of Bioline Hyperladder1 (Bioline, Alexandria, NSW, Australia) was added to the first well of the gel the containing samples, the size and concentrations of the bands were assessed by comparison to the ladder (Figure 2.1). The sizes of DNA fragments amplified in each PCR reaction are listed in Table 2.2.

For visualising the gels, a Chemi Genius2 BioImaging System (Syngene, Cambridge, UK) transilluminator was used. The gel images were visualised, analysed, and recorded using the Gene Snap image acquisition software (Synoptics, Cambridge, UK).

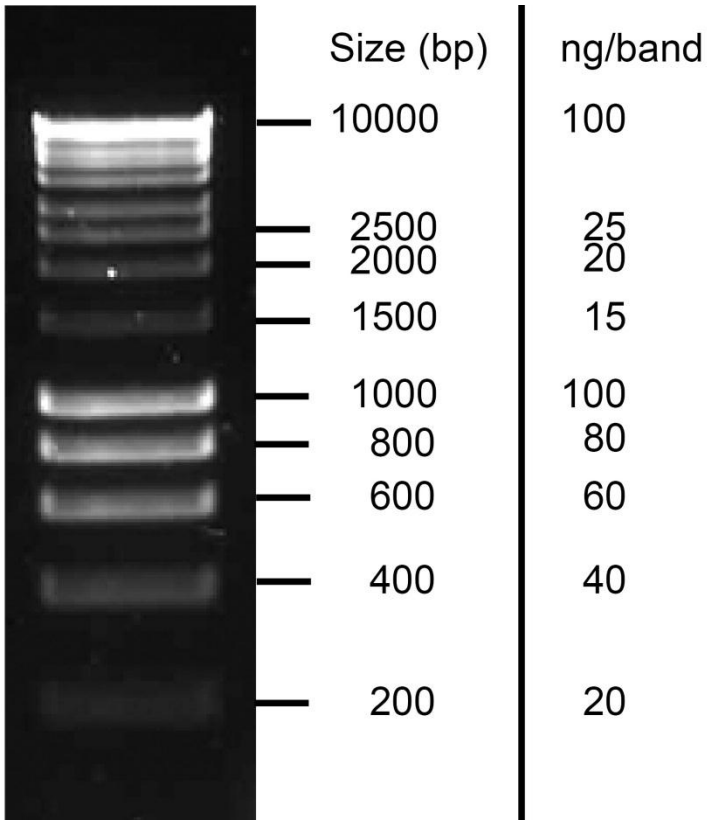


**Figure 2.1.** Hyperladder I band sizes. The 14 bands indicate specific DNA lengths and variation in band intensity relate to the loadings of each fragment in nanograms. Fragments in this study all ranged between 200 - 2000 bp, so hyperladder I was the only DNA ladder used in this study.

### 2.2.6 Gel purification of PCR products

PCR products were cleaned prior to sequencing. DNA PCR products were run on an agarose gel until bands were sufficiently separated. A Dark Reader (UV) transilluminator (Clare Chemical Research, Dolores, Colorado, USA) was used to visualise DNA bands while these were excised with a scalpel. The DNA was extracted from the gel using the Mega-Spin agarose gel extraction kit (Intron Biotechnology, Gyeonggi-do, Seoul, South Korea) following the manufacturer's instructions. This kit uses a MegaSpin column to trap DNA while washing through impurities. DNA was then resuspended in elution buffer from the extraction kit.

### 2.2.7 Sequencing

For confirming the positioning of the T-DNA inserts in the gene of interest, sequencing of the PCR products was required. PCR products for sequencing were sent as purified DNA along with NanoDrop concentration values (ng/µl) and PCR primers (10 µM concentrations). Sequencing was performed at Macrogen Korea (Geumcheon-gu, Seoul, South Korea).

### 2.2.8 Bioinformatic analysis

The PCR product sequences were screened using the BLAST online program comparing the PCR product sequences to the *Arabidopsis thaliana* sequence database. The sequence data was aligned to the homolog sequences using the nucleotide BLAST engine.

Nucleotide BLAST sequence alignment tool was used to determine the upstream and downstream junctions of the T-DNA insert. The PCR product sequences were aligned against the *A. thaliana* genome sequence and the T-DNA insert vector sequence and determined the junctions from the PCR product sequence, determining the insertion site.

## 2.3 *Arabidopsis* knockout phenotyping

### 2.3.1 Whole plant growth development analysis

T-DNA knockout *A. thaliana* plants were planted alongside wild-type Columbia ecotype lines to assess any visible phenotype in plant structure and development. The progeny of knockout lines that were confirmed homozygous for the T-DNA insert were harvested and planted with wild-type controls in growth rooms 1 and 2. For each line there were 9 pots in a large clear

container in both rooms. They were watered regularly and a cover was put around the plant once it had started to bolt.

The phenotype log for the plants included:

i) when plant bolted and flowered,
ii) the number of leaves when plant bolted and flowered,
iii) the height of inflorescence at flowering, and
iv) any other phenotype related notes.

This log was compiled over the six week *A. thaliana* life cycle.

The numbers of days and leaves were calculated for each plant. The statistical analysis and graphing the phenotype numbers for each line were performed using GraphPad Prism 6 (GraphPad Software, La Jolla, California, USA). One-way ANOVA and Dunnett's multiple comparisons test to the wild-type line were performed in analysing knockout lines. Chi-Square analysis was used in determining statistical significance of the observed distribution of F2 double knockout progeny to the expected distribution frequencies.

### 2.3.2 Seedling Root Growth Measurements

Root growth was compared in knockout lines and wild-type lines. After 5 days of growth on agar, six seedlings from each line being tested were transferred onto new agar plates. Scans of the seedlings were collected using transmitted light with an Epson Perfection V700 scanner (Suwa, Nagano, Japan) at 0 h, 24 h, 48 h, and 78 h time points, and measured using the ruler tool in Adobe Photoshop CS6 (Adobe, San Jose, California, USA).

A positive control of root growth inhibition in *Arabidopsis* seedlings was tested with oryzalin, a herbicide drug involved in microtubule depolymerisation and blocking of anisotropic growth in plant cells. The mutant line *mor1-1*, known to display root growth inhibition under oryzalin concentrations (Collings et al. 2006), was compared to wild-type and Nup knockout lines. Five oryzalin concentrations (0, 33, 100, 330, and 100 nM) were tested with six seedlings of each line. To make the plates, oryzalin (Chem Service, West Chester PA, USA, 0.5 mM stock in DMSO) was added to liquid molten agar before pouring of media for each concentration. The *mor1-1* mutant is temperature sensitive and displays exaggerated grown inhibition at 30°C. Two replicates of every concentration were placed in separate growth cabinets of 21°C and 30°C. GraphPad Prism 6 program was used to create graphs and

statistically analyse root elongation measurements of knockout lines with a one-way ANOVA and Dunnett's multiple comparisons test to the wild-type line measurements.

### 2.3.3 Leptomycin B growth inhibition assay

Leptomycin B (LMB) is an inhibitor of nuclear transport, and its activity can be assayed using fluorescent nuclear transport proteins (such as GFP-PUM) that will accumulate in the nucleus on addition of LMB (Tam et al. 2010). LMB was purchased from LC Laboratories (Woburn, Massachusetts, USA) as a 1 mM stock solution in absolute ethanol (100 µg in 185 µl). Working stock solutions were prepared in ethanol and stored at -20°C. In testing for LMB effectiveness, onion epidermal cells and *Arabidopsis* epidermal cells in young seedlings were transformed by particle bombardment (see section 2.4) with GFP-PUM, RFP-PUM, and control constructs. Plant tissue expressing GFP was floated on 2 ml of water containing 370 nM of LMB as onion epidermal cells transiently expressing GFP-PUM displayed nuclear GFP accumulation while floated on this concentration (Tam et al. 2010). The effect of the drug was initially checked with a stereo-fluorescence microscope after 5 h, and after 24 h, onion and *Arabidopsis* cells transformed with GFP-PUM, RFP-PUM, and control constructs were imaged with confocal microscopy (see section 2.5.3).

Multiple different tests were used to determine whether LMB modulates *Arabidopsis* growth:

i) 5 d old *Arabidopsis* seedlings were transferred onto different agar media containing LMB (0, 3.3, 10, 33, and 100 nM). LMB is known to inhibit the nuclear transport of *Arabidopsis* leaf tissue at 10 nM concentration (Tillemans et al. 2006). 0.1 mM of LMB stock diluted in absolute ethanol was added to liquid molten agar before pouring of media for each concentration. Six seedlings for each *Arabidopsis* line of wild-type and Nup knockout lines were transferred to each concentration.

ii) As no effect on growth was seen when high concentrations of LMB was dissolved in agar, the stability of LMB in agar came into question, as LMB is unstable in compounds such as DMSO (Asscher et al. 2001). A second test was designed to determine whether the LMB had been inactivated by dissolution into molten agar. The 5 d old test seedlings for the second test were transferred onto filter paper soaked in two concentrations of LMB (0 nM and 2000 nM) diluted in distilled water. The filter paper in the plate was in contact with the remaining solution at the bottom of the plate to avoid drying.

In both experimental systems, plates were scanned after the transfer (0 h) and again at time points 24 h, 48 h, and 72 h afterwards. The same procedure in root measurements and statistical analysis were carried out in seedling root growth measurements (see section 2.3.2).

### 2.3.4 Nucleocytoplasmic transport analysis

The ratio of the fluorescent intensities in the nucleus and cytoplasm of onion and *Arabidopsis* cells transiently expressing RFP-PUM or GFP-PUM represents a measure of nucleocytoplasmic transport of proteins within the cell. The fluorescence of the cells from captured images by confocal microscopy were measured by quantifying the mean grey pixel values from the areas of fluorescence in the nucleus and cytoplasm. These values were obtained using the program LAS AF Version 2.6.0 build 7266 (Leica, Wetzlar, Germany).

The polygon tool in the program was used to mark the nucleus and an area of cytoplasm. The mean grey values were then measured. Another area that contained the lowest amount of fluorescence in the image was selected and measured as a background value, and this value was deducted from nucleus and cytoplasmic measurements. The nucleus to cytoplasmic fluorescent ratio was then calculated. GraphPad Prism 6 program was used to create graphs and statistically analyse ratio values with a one-way ANOVA and Dunnett's multiple comparisons test to the wild-type line. For statistical analysis of leptomycin B treatments for each plant line, an unpaired t-test was used in comparing treated and untreated cells.

## 2.4 Transient expression assays by particle bombardment

### 2.4.1 Plasmid miniprep for particle bombardment

The details and source of plasmid constructs used in particle bombardment experiments are listed in Table 2.3. The expressed free YFP and PUM fusion constructs localise to the nucleus and cytoplasm (Lechner et al. 2012; Tam et al. 2010). The DNA for plasmid miniprep was transformed into competent *Escherichia coli* cells. 1 µl DNA or a piece of dried filter paper (~5 $mm^2$) containing DNA was added to an Eppendorf tube containing competent *E. coli* cells and incubated on ice for 20 min. The cells were heatshocked at 42°C for 2 min and the total volume was spread across an agar plate (1% tryptone w/v, 0.5% yeast extract w/v, 1% NaCl w/v, 1.5% Agar w/v) containing ampicillin 100 µg/ml (DNA contains ampicillin resistance gene marker). The cultured plate was incubated at 37°C overnight. Bacterial colonies growing on the media were transferred into a 10 ml liquid LB (1% tryptone w/v, 0.5% yeast extract

w/v, 1% NaCl w/v, pH 7.0) and incubated overnight with shaking at 37°C. The next day a miniprep was conducted of the overnight LB-culture using the PureLink Quick Plasmid Miniprep Kit (Life Technologies, Auckland, New Zealand). The miniprep was conducted following the manufacturer's instructions, and resulted in purified plasmid DNA resuspended in 75 µl TE buffer. DNA concentration (ng/µl) was collected by Nanodrop. Glycerol stocks were also prepared from 500 µl LB -culture that was added to 500 µl of glycerol for cryostorage at -80°C.

### 2.4.2 Gene gun particle bombardment transformation

Transient transformation was used to observe cellular dynamics in plant tissues. This transformation of plant tissue samples was performed with particle bombardment, consisting of plasmid DNA constructs (encoding fluorescent reporter protein) precipitated onto gold particles and bombarded into the plant tissue. Cells in which a particle lodged into the nucleus were induced to express the foreign DNA. Particle bombardment transformation was done using a helium-powered gene gun (Kiwi Scientific, Levin, New Zealand) within a vacuum chamber.

Gold particles 1.0 µl or 1.6 µl in diameter were purchased from BioRad (Hercules, California, USA) and were washed in distilled water and 100% (v/v) ethanol multiple times before being resuspended in distilled water. Aliquots containg 1 mg of gold particles in 25 µl of distilled water were prepared and stored at -20°C.

To coat DNA onto the gold particles, the particles were resuspended by running the tubes along a hard plastic Eppendorf rack. Once the gold particles were resuspended, 5 µl of plasmid DNA (approx. 50 ng/µl concentration) was added, the particles vortexted, and 25 µl of sterile 2.5 M calcium chloride added to precipitate DNA. After vortexing, the DNA was stabilised by addition of 10 µl of 0.1 M spermidine, the particles were resuspended by vortexing. The sample was then spun down on a bench top microfuge and the supernatant was removed. After washing in 180 µl of 100% (v/v) ethanol, the DNA-coated gold particles were pelleted and resuspended in 100 µl of 100% (v/v) ethanol.

Gene gun shootings were performed in a biosafety cabinet. Samples were placed on a shelf within the gun chamber at approximately 7 cm from the nozzle. For onion samples, plant tissue was excised from the third of fourth layer of the bulb. The sample was placed on moistened tissue on a 90 mm diameter plastic Petri dish and put in the chamber with the

mesophyll layer facing up. For *Arabidopsis* samples, multiple seedlings (20-30) were placed on a moistened tissue on top of Styrofoam sitting on a 90 mm diameter petri dish. These seedlings were covered with a metal mesh that was then pinned into the underlying Styrofoam. Preparation of *Arabidopsis* seedlings for gene gun bombardment is displayed in Figure 2.2.

Plasmid DNA-coated gold particles were resuspended and 10-20 µl was applied to a Swinnex filter and dried for 20 s. The filter was installed in the gene gun chamber. The firing timer on the gene gun control box was set to 40 milliseconds, and a 60 psi pulse of helium gas used to fire the particles into the tissue once a vacuum had been pulled.

After shooting the samples were covered in moistened tissues, sealed and stored for 24-48 h to allow expression of fluorescent proteins.

**Table 2.3.** Plasmids used in particle bombardment experiments.

| Code | Description | Source |
|---|---|---|
| GFP-PUM | GFP fusion to PUM Protein | Doug Muench, The University of Calgary, Alberta, Canada (Tam et al. 2010). |
| RFP-PUM | RFP fusion to PUM Protein | Doug Muench, The University of Calgary, Alberta, Canada (Tam et al. 2010). |
| YFP | YFP Protein | Madeleine Rashbrooke, the Australian National University, Canberra, ACT, Australia (Lechner et al. 2012). |

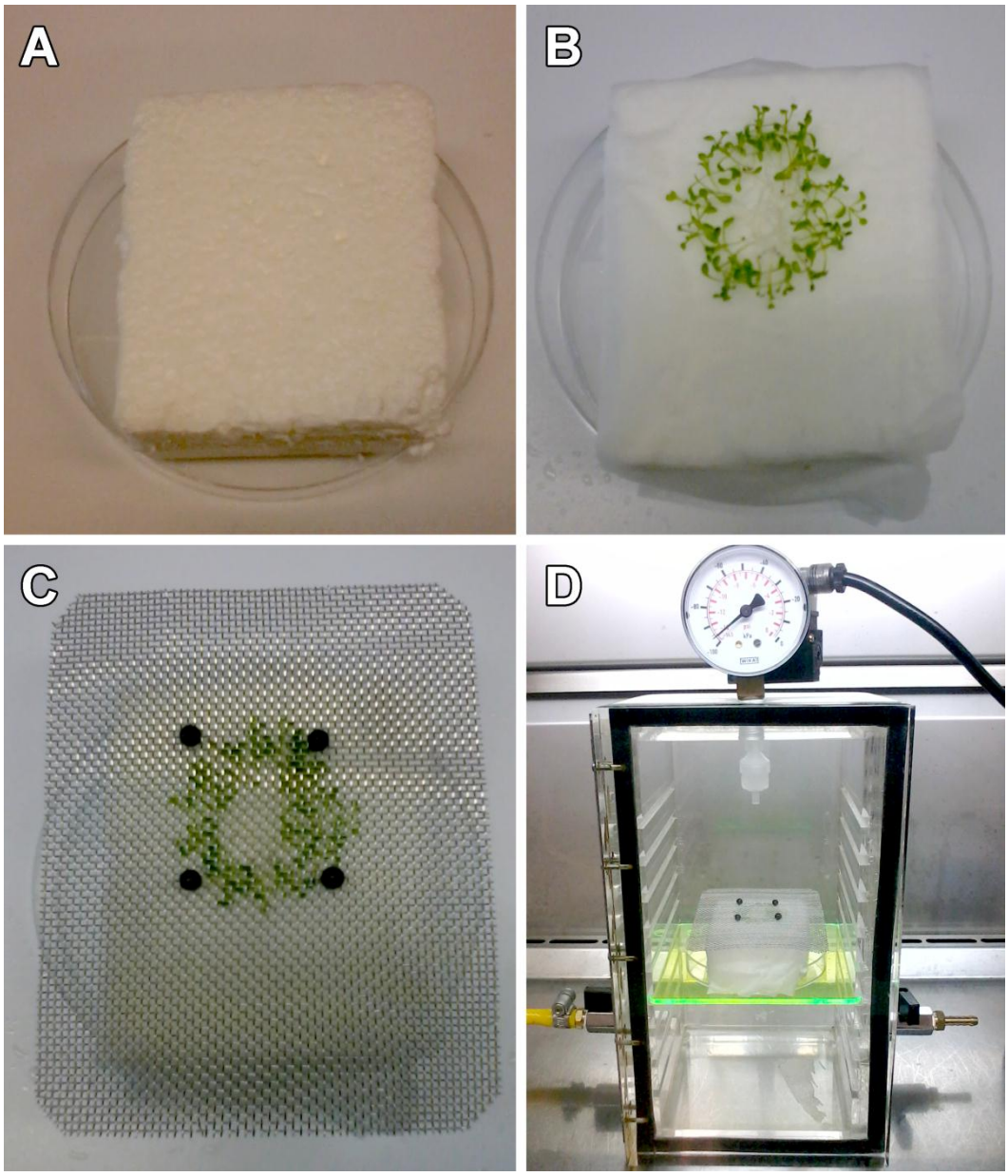


**Figure 2.2.** Preparation of *Arabidopsis* seedlings for transformation by gene gun bombardment.

**A** Styrofoam placed in culture plate.

**B** *Arabidopsis* seedlings arranged on wet tissue paper over styrofoam.

**C** Metal mesh pinned over seedlings and into styrofoam.

**D** Sample placed into vacuum chamber for particle bombardment.

## 2.5 Microscopy

### 2.5.1 Light microscopy

*Arabidopsis* stem inflorescence sections of 50 µm were cut using a Vibratome 1000 Plus (Intracel, Hertfordshire, UK). These sections were stained with 0.1% (w/v) toluidine blue to visualise tissue structures (Parker et al. 1982). A light microscope (Nikon, Tokyo, Japan) using a 4X magnification lens was used to observe sections, and a Leica DFC310 FX Camera (Leica, Wetzlar, Germany) recorded images.

### 2.5.2 Stereo-fluorescence microscopy

Transformed plant cells were initially screened with a stereo fluorescence dissecting microscope equipped with a mercury lamp and fluorescent optics (Leica MZIOF, Wetzlar, Germany). Excitation and emission filters were:

i) UV excitation and long pass (visible light) emission filter.
ii) Blue excitation and a long pass (green plus red) emission filter. This combination was used for GFP and YFP.
iii) Green excitation and a red band pass emission filter. This combination was used for RFP.

Samples transformed with GFP, YFP, and RFP would be screened visually before being transferred to the confocal microscope for imaging.

### 2.5.3 Confocal microscope imaging

Fluorescence and concurrent transmitted light images were recorded in optical section and time series modes with a confocal microscope (Leica SP5, Wetzlar, Germany) with 20X glycerol immersion and 40X oil-immersion lenses. The line averaging of all images was set at 8 and z-sections had a step size of 2 µm. The laser excitation wavelengths used were 488 nm for GFP, 514 nm for YFP, and 561 nm for RFP. The fluorescence of samples were imaged with emissions of 510 nm-550 nm for GFP, 525 nm-600 nm for YFP, and 575 nm-650 nm for RFP. For dual labelling experiments of GFP and RFP, sequential scanned images were collected separately to avoid fluorescent overlap of both proteins. All images were processed with LAS AF Version 2.6.0 build 7266 and Adobe Photoshop CS6.

# Chapter 3

# T-DNA Screening and Sequencing

## 3.1 Introduction

### 3.1.1 *Agrobacterium* T-DNA transformation

The ubiquitous soil bacterium *Agrobacterium* infects a wide variety of plants, causing diseases crown gall, cane gall, and hairy root (Gelvin 2009). The agent that causes the crown gall tumor is a small region of transferred DNA (T-DNA) that originates from a Ti-plasmid, and which is inserted into the infected plant's genome (Chilton et al. 1977). The inserted T-DNA encodes for genes involved in the synthesis of auxin and cytokinin that are the driving factors in the tumor causing neoplastic growth characteristic of the disease (Akiyoshi et al. 1984; Schröder et al. 1984).

*Agrobacterium tumefaciens*-mediated transformation is the most widely used method for introducing genes into plants and subject to numerous studies over the past few decades (reviewed inTzfira and Citovsky 2006). Because only the left and right borders sequences of the T-DNA are required for the transfer of the T-DNA into the plant, the large part of the T-DNA sequence can be replaced with foreign genetic material (Wang et al. 1984). The recombinant strains of *Agrobacterium* used in transformation have, therefore, had their native T-DNA replaced with other genes of interest and are vehicles for inserting foreign DNA into the genome to produce transgenic plant species (Nester et al. 2005).

Because the insertion of the T-DNA occurs randomly through the plant genome, it has also been recognised that insertional events into the open reading frame or promoter of a gene can directly disrupt the function of that gene (Feldmann et al. 1989; Koncz et al. 1990). The generation of gene knockouts through this approach is referred to as insertional mutagenesis.

The development of this technique of insertional mutagenesis has been a key development in studies of gene function in the model plant *Arabidopsis* (Koornneef and Meinke 2010). In the traditional forward genetics approach that has been refined over the last century, observations of an altered phenotype are used to find the altered genotype. Reverse genetics is the opposite approach to characterising gene function, and begins with a mutant gene sequence and determines that resulting change in phenotype. Three key developments have allowed reverse genetics to become the main approach to characterising the entire genome (Krysan et al. 1999). These developments have been:

i) the completed genome sequence for *Arabidopsis*,
ii) the advent of insertional mutagenesis, and,
iii) the development of the 'floral dip' method that has greatly simplified the once laborious task of *Agrobacterium* transformation of *Arabidopsis* (Clough and Bent 1998).

Reverse genetics has several important advantages over forward genetic approaches. This is because forward genetic approaches will generally fail to expose a phenotype when more than one gene is required for disruption. In reverse genetics, however, crosses between plants containing T-DNA insertions in closely related genes can be used to systematically test for redundant gene functions (O'Malley and Ecker 2010).
T-DNA insertion lines have confirmed gene function in an enormous variety of biological processes, including, cell wall biosynthesis and structure (Brown et al. 2005), disease resistance (Knoth et al. 2007), floral development (Wellmer et al. 2004), metabolic regulation (Hussain et al. 2004), hormone synthesis (Staswick et al. 2005), hormone signalling (Chini et al. 2007), and mRNA regulation (Gasciolli et al. 2005).

A very large number of independently-transformed seed lines are required to saturate the genome in order identify insertions in any particular gene (Krysan et al. 1999). *Arabidopsis* as a reference plant with the most accessible resource in T-DNA mutant collections plays an important role in ultimately defining traits for agricultural plants. Collections of T-DNA insertional mutants in *Arabidopsis* have been produced by several laboratories. These collections have been designed so that pooled lines that can be probed by PCR using primers specific to the gene and T-DNA to search for inserts in the gene of interest (Krysan et al. 1999; McKinney et al. 1995). Knockout libraries containing over 150,000 T-DNA insert lines for *Arabidopsis*, have been created and catalogued through the *Arabidopsis* Information

Resource Database (TAIR; www.arabidopsis.org). This collection enables researchers to directly order seed lines with a T-DNA insert within their gene of interest for study. These lines include the SALK unimutant collection consisting of 31, 033 total homozygous lines that have been identified and made public. These lines represent 18, 506 individual genes (O'Malley and Ecker 2010). The availability of two independent alleles for each gene is critical in allowing an observed phenotype to be immediately confirmed to prevent false positive gene function annotations (O'Malley and Ecker 2010).

### 3.1.2 Screening for knockout lines and determining the insert site

When screening for gene knockout lines, T-DNA-containing lines have been recovered and enriched by positive selection for antibiotic resistance markers located within the T-DNA vector (Pan et al. 2005). However, approximately 30% of T-DNA transformation events result in non-expressing transgenic markers in which antibiotic resistance marker has been silenced. This silencing prevents the identification of knockouts by selection protocols such as the use of kanamycin-selected transformants (Francis and Spiker 2005). Furthermore, antibiotic screening methods do not effectively discriminate between plants that are homozygous for the gene knockout and those that are heterozygous for the knockout, for in these plants, expression of the resistance marker can occur along with functionality of the knocked out gene (Su and Li 2008). Therefore, PCR-based methods have been developed for isolating individual plants that are heterozygous and homozygous for particular T-DNA insertional events (Krysan et al. 1996; McKinney et al. 1995). Using the proper PCR strategy, the presence of a T-DNA insertion within any given gene can be detected (Krysan et al. 1999). Also because T-DNA inserts are associated with each gene knockout, genotyping complex mutant backgrounds is possible through PCR (Krysan et al. 1999).

The upstream and downstream junctions of the T-DNA insertion can be determined by directly sequencing the PCR products, thereby determining the site of the T-DNA mutation (McKinney et al. 1995). Alternatively, by sequencing the T-DNA/genomic junction from the PCR product, the precise location of the insert can be mapped against the reference (O'Malley et al. 2007). PCR is the most effective technique in screening for homozygote knockout lines over antibiotic screening methods as the T-DNA insert sequence also provides a target for a PCR primer to map the precise location of the insertion site (O'Malley et al. 2007).

### 3.1.3 Using knockout lines to study the nuclear pore complex

T-DNA insertion mutant lines have been used to determine the physiological role of *Arabidopsis* nuclear pore complex proteins (reviewed in Meier and Brkljacic 2009). Early flowering phenotype and pleiotropic developmental defects including smaller plant size, abnormal phyllotaxy, terminal floral structures and reduced fertility have been exhibited in Nup mutants *sar3, sar1, attpr,* and *nup136* (Dong et al. 2006; Jacob et al. 2007; Parry et al. 2006; Tamura et al. 2010). The pleiotropic defects in *sar1* and *sar3* single mutants were also exaggerated in *sar1 sar3* double mutants, demonstrating a further disruption of NPC general or specific function with the loss of multiple subunits (Parry et al. 2006). Molecular phenotypes such as calcium spiking and nuclear mRNA accumulation have also been described in the Nup mutants *sar1, sar3, nup85, nup133,* and *attpr* suggesting a role for Nups in mRNA export and transducing a calcium signal at the NPC (Meier and Brkljacic 2009). Thus, T-DNA mutants of the NPC have helped define NPC structure and uncover molecular and developmental phenotypes.

Transmembrane nuclear pore anchoring proteins have been more extensively investigated in vertebrates and fungi than in plants. Experiments that have involved knocking out these anchoring proteins have previously been conducted in vertebrates and fungi and have contributed to defining the function of these Nups. The elimination of NDC1 in the nematode *Caenorhabditis elegans* resulted in NPC assembly defects and very high larval and embryonic mortality (Stavru et al. 2006a). NDC1 was also found to be essential to mitotic viability in the yeast *Saccharomyces cerevisiae* with conditional lethality in mutants. It was deduced to be a component of the spindle pole body as well as the NPC body (Winey et al. 1993). In mouse C2C12 myoblasts and embryonic stem cells strong cell death is associated with down-regulation of GP210 with RNAi, while under differentiation conditions, GP210 was found to regulate induction of genes essential for differentiation (D'Angelo et al. 2012). The use of T-DNA knockout mutants containing inserts located in *Arabidopsis* genes homologous to vertebrates and fungi would for the first time describe these Nup proteins responsible for anchoring the NPC in higher plants.

### 3.1.4 Objective

In characterising *NDC1* and *GP210* loss-of-function in *Arabidopsis*, *ndc1* and *gp210* homozygous knockout lines were isolated from seed stock by PCR screening and

subsequently selfed for homozygous progeny. The PCR products from knockout screening were purified for sequencing. Sequencing data was put through BLAST alignment to determine the insertion site from the junctions of alignment to the *Arabidopsis* genome and T-DNA insert in the PCR product.

Two homozygous knockout lines, *ndc1* and *gp210*, were isolated from PCR screening for further growth and development characterisation. Sequencing and BLAST analysis confirmed the T-DNA insertions of both knockout lines to located in the exon region of both homolog *Arabidopsis* Nup genes, confirming gene knockout.

## 3.2 Results

### 3.2.1 PCR screening of knockout lines

Due to possible gene silencing of antibiotic markers and the need for more accurate genotyping, a PCR screening protocol was favoured over positive selection for antibiotic resistance markers (Francis and Spiker 2005; Krysan et al. 1999). PCR was conducted to isolate lines homozygous for T-DNA inserts in the genes At1g73240 and At5g40480.

Seed stock received from the *Arabidopsis* Biological Resource Center (Ohio State University) was germinated, planted on soil and DNA subsequently extracted from mature leaves. This DNA was added to PCR reaction mixtures containing their respective primers to identify homozygous plants. The primers were left and right border genomic specific primers and a T-DNA insert primer (Table 2.2). DNA elongation between the genomic primers occurred when no insert was present and DNA elongation between the T-DNA specific primer and the right border genomic primer occurred when the T-DNA insert was present at the gene location (Figure 3.1). In isolating homozygous lines, PCR reaction mixtures originally contained all three primers, but it was observed that heterozygotes were not effectively being distinguished from homozygotes so an alternative protocol using two PCR reactions for each sample was used (Ajjawi et al. 2010). One reaction would contain the left and right border genomic primers, and the other would contain the right genomic primer and the T-DNA insert primer (Figure 3.2).

The PCR products were separated through agarose gel electrophoresis, and the genotype of plant determined by a PCR fragment produced in each reaction (Figure 3.2). Shorter fragments run down the gel faster than longer ones so bands further down the gel are shorter

than the bands above. Since the amplified region between the genomic border primers was larger than the region between the T-DNA insert and right border genomic primer, a larger PCR fragment product was formed in non-T-DNA containing wild-type samples. T-DNA insert containing lines had the shorter fragment. No band was produced for wild-type samples in the insert primer and right border primer PCR reaction, as no insert was present. In homozygous lines no band was produced from the PCR reaction containing both genomic primers, as there was a T-DNA insert present at both alleles disrupting elongation between primers. Heterozygous lines produce fragments for both PCR reactions, since there the T-DNA insert was present in one allele.

There were four plant lines originally tested for knockout screening, each contained a T-DNA insertion at different regions of At1g73240 and At5g40480 (Table 2.1). The two plant lines used for screening *ndc1* were T65 and T79; the plant lines used for *gp210* were T74 and T83. Homozygous lines using T74 and T79 were not isolated as no knockout bands were observed in PCR screens. However, as homozygous knockout lines of *ndc1* and *gp210* were successfully isolated from T65 and T83 plant lines, for use in this study. Further screening of T74 and T79 plants by redesigning primers for inserts was not conducted. Homozygous knockout lines *ndc1* and *gp210* were confirmed by PCR (Figure 3.3). The wild-type band for screening *NDC1* was 1080 bp in length, the knockout length was 642 bp. For the *GP210* screening the wild-type band length is 1232 bp, the knockout length was 747 bp.

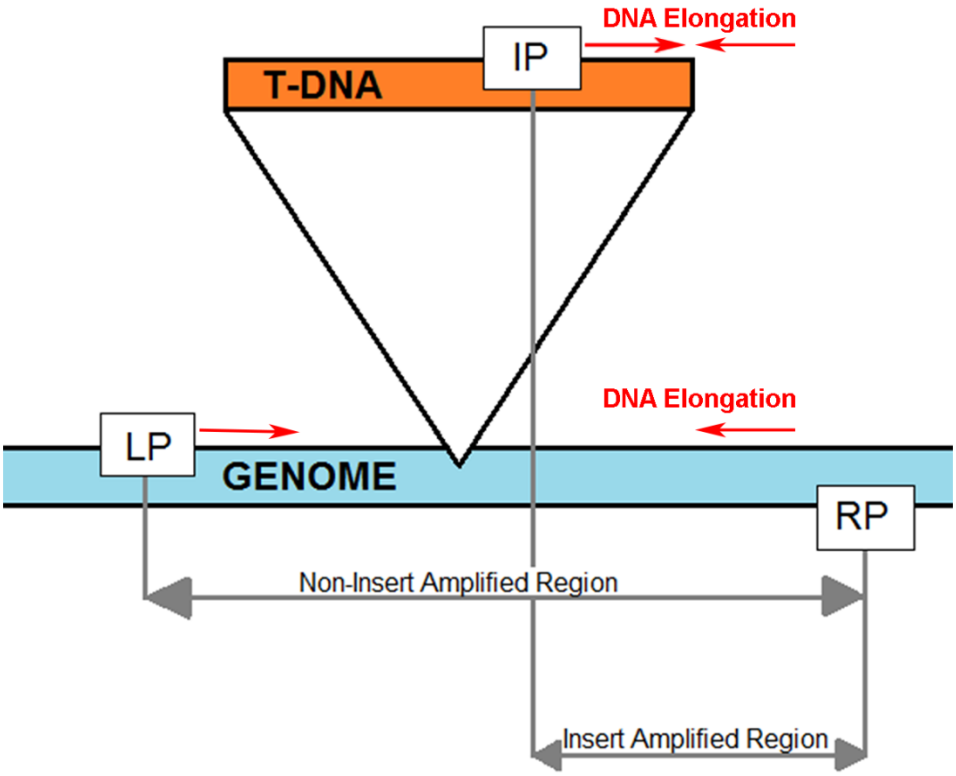


**Figure 3.1.** PCR screening primer locations of T-DNA knockout lines. The insertion of the T-DNA into the genome causes disruption of DNA elongation between the genomic primers. DNA elongation between the right genomic primer and the insert primer targeting the T-DNA sequence creates a shorter DNA fragment than a sample containing no T-DNA insert thus allowing detection of knockout lines.

**IP** T-DNA Insert primer

**LP** Left genomic border primer

**RP** Right genomic border primer

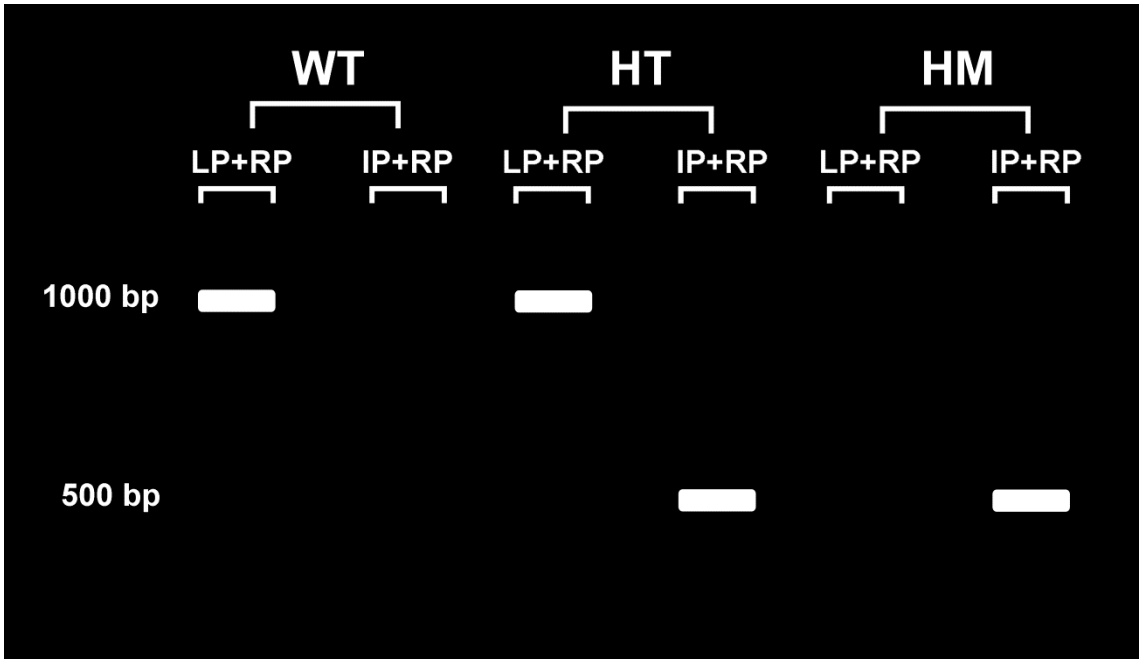


**Figure 3.2.** Identification of knockout lines by PCR screening. Wild-type (WT) lines will only produce a DNA fragment from the genomic primers. Homozygous (HM) lines will only produce a shorter DNA fragment from the insert primer and the right genomic primer. Heterozygous (HT) lines will produce fragments from both pairs of primers.

**LP+RP** PCR reaction containing left and right border genomic primers.

**IP+RP** PCR reaction containing insert and right border genomic primers.

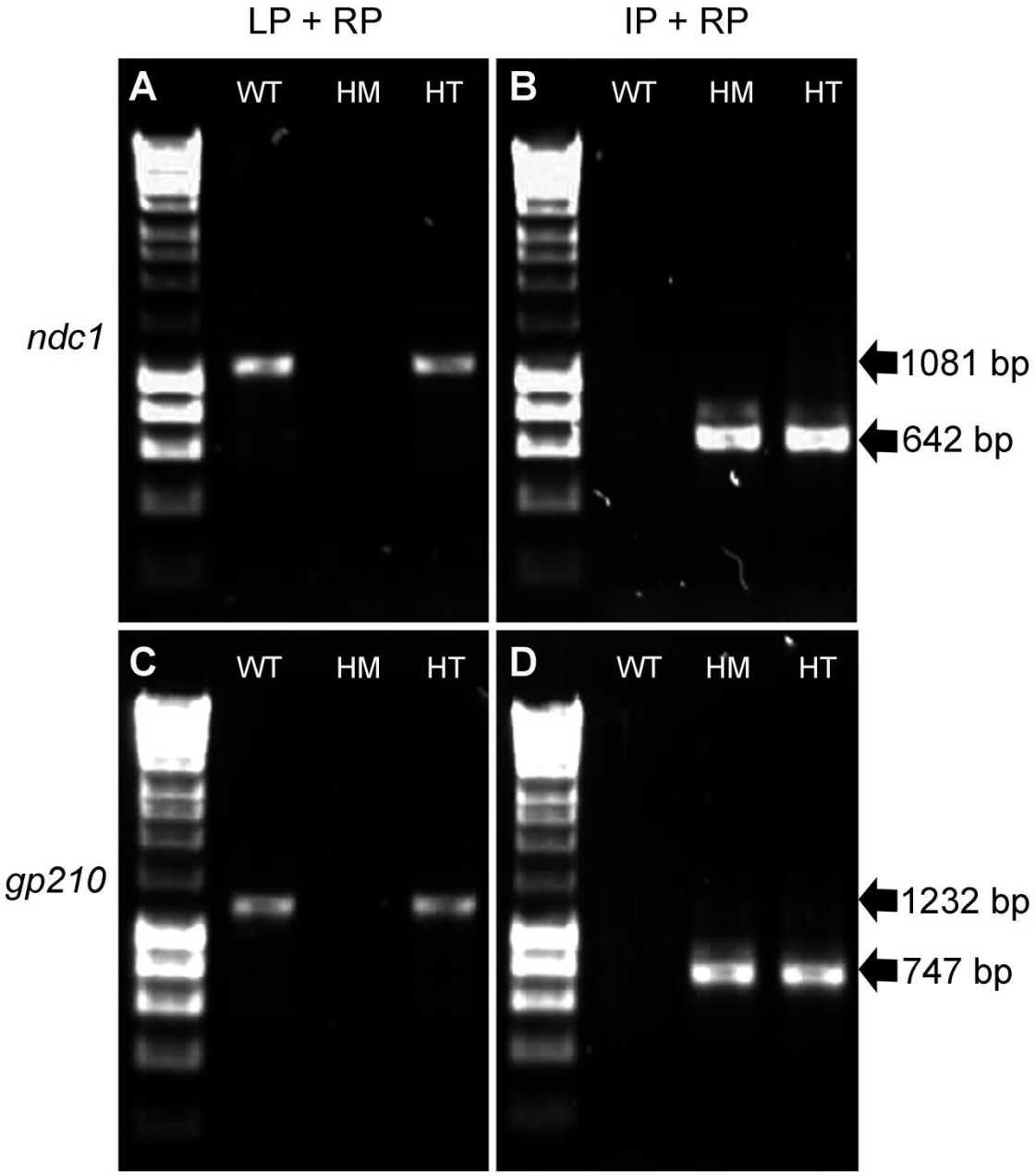


**Figure 3.3.** PCR screenings for *ndc1* and *gp210* knockout lines:

**A** *NDC1* PCR screen using left and right genomic primers (LP + RP).

**B** *NDC1* PCR screen using insert primer and right genomic primer (IP + RP).

**C** *GP210* PCR screen using left and right genomic primers (LP + RP).

**D** *GP210* PCR screen using insert primer and right genomic primer (IP + RP).

Sample DNA was identified as wild-type (WT), homozygous (HM) or heterozygous (HT) from both PCR reactions; LP + RP and IP + RP. Arrows indicate band sizes on gel.

### 3.2.2 Sequencing PCR products

In determining the precise locations of the T-DNA insertions, PCR products can be sequenced and mapped against the *Arabidopsis* reference genome, thereby defining the upstream and downstream junctions of the T-DNA insertion (McKinney et al. 1995). The PCR products the bands in the gels were excised, purified (Figure 3.4) and sent for sequencing. Before shipping, the samples for sequencing the concentration of the purified DNA products was determined by Nanodrop to ensure sample concentration was above the required concentration for sequencing.

Nucleotide sequence data were analysed through BLAST online sequence alignment (http://blast.ncbi.nlm.nih.gov/Blast.cgi) against the *A. thaliana* genome. The sequence of wild-type PCR product from *NDC1* screening aligned with the gene At1g73240 (Appendix 1.1). The BLAST alignment confirmed that the wild-type contained no T-DNA insert in the *NDC1* gene and that there was a 100% sequence agreement with the published *Arabidopsis* genome sequence for the Columbia ecotype. The wild-type PCR product sequence from *GP210* screening aligned with the At5g40480 (Appendix 1.2) with 98% sequence identity with published Columbia genome, confirming no T-DNA insert present in the *GP210* gene.

In defining the location site of the T-DNA insertion, the PCR product sequence from the homozygous knockout band was aligned against the *Arabidopsis* genome and the T-DNA insert vector sequence. This determined the *Arabidopsis* genome and T-DNA junctions of the knockout PCR product. The knockout PCR product sequence from the *NDC1* screening aligned with the At1g73240 gene with 99% sequence agreement (Appendix 1.3). The knockout PCR product was also aligned with the SAIL insert sequence with 99% sequence agreement (Appendix 1.4). The *Arabidopsis* genome junction and T-DNA junction of the knockout PCR product was determined from these alignments (Appendix 2.1). The T-DNA insertion point on the *NDC1* gene was determined to be at 27, 544, 410 bp in the exon region of chromosome 1 (Figure 3.5A), this was within the range along the gene indicated on the TAIR database.

The knockout PCR product from the *GP210* screening aligned with the At5g40480 gene with 99% sequence agreement (Appendix 1.5). The knockout PCR product also aligned with the SALK insert sequence with 99% sequence agreement (Appendix 1.6). The *Arabidopsis* genome and T-DNA junctions of the *GP210* knockout PCR product was

determined (Appendix 2.2). The site of T-DNA insertion in the GP210 gene was determined to be at 16, 215, 860 bp in the exon region of chromosome 5 (Figure 3.5B).

## 3.3 Conclusions

Homozygous knockout lines of At1g73240 and At5g40480 genes homologous to *NDC1* and *GP210* were successfully isolated through PCR screening. The sites of the T-DNA insertions were confirmed through the sequencing of PCR screening product. The sequences allowed the mapping of the T-DNA insertions within both targeted homologous genes through the alignment of the PCR products to the *Arabidopsis* genome and T-DNA vector sequences. The T-DNA insertions were confirmed to be located within gene-coding exon regions as described by the TAIR database. Both knockout lines contain T-DNA insertions mapped within the translated exon region of the gene confirming a very high probability of gene disruption (Wang 2008). With isolated homozygous knockout plant lines targeted to *NDC1* and *GP210* homolog genes further analysis of the genes can be performed by investigating the plant development and cellular dynamics using *ndc1* and *gp210* knockout lines.

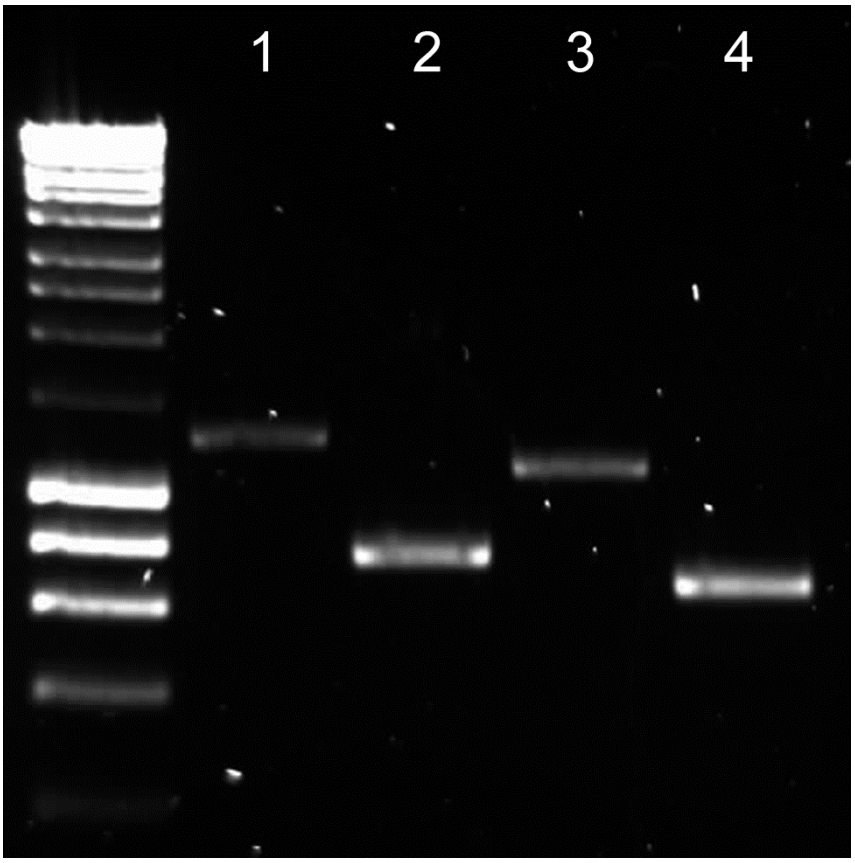


**Figure 3.4.** DNA purified from gel extraction kit for sequencing. Loadings were determined from comparing samples to the gel ladder, concentrations were determined by comparing band intensity to the ladder (ng/ml), from loading volume. Gel lanes were:

**1** *GP210* wild-type 50 ng.

**2** *gp210* homozygous knockout 70 ng.

**3** *NDC1* wild-type 55 ng.

**4** *ndc1* homozygous knockout 80 ng.

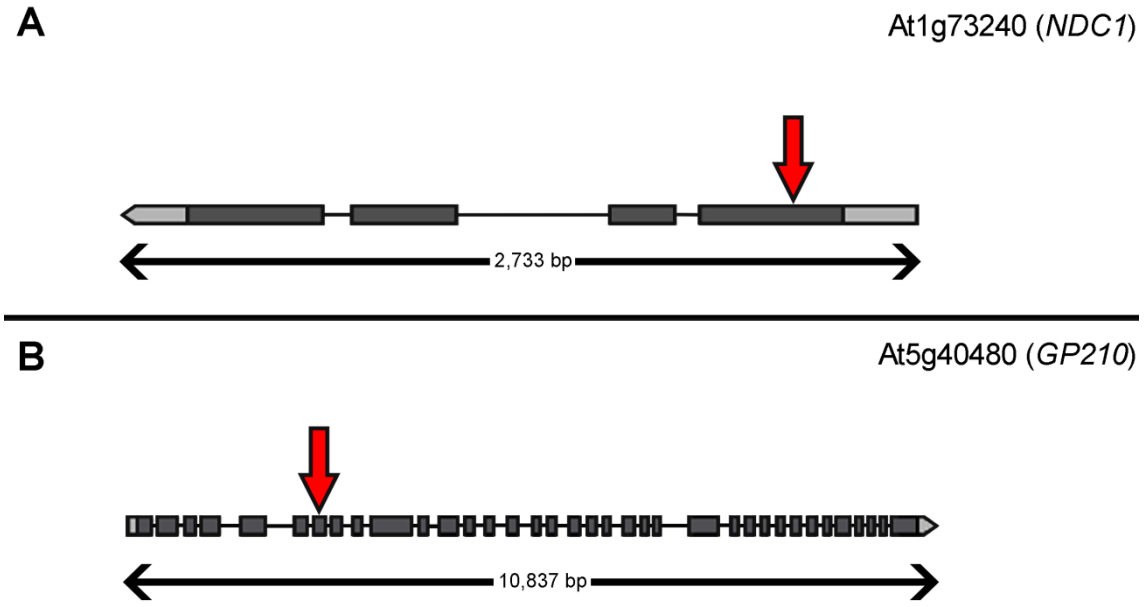


**Figure 3.5.** Gene homolog size, structure and location of T-DNA insert:

**A** *NDC1* locus At1g73240.

**B** *GP210* locus At5g40480.

Gene models pointing right or left indicate forward or reverse directed transcription of gene respectively. Exons are displayed as boxed regions and introns are line regions. Protein translated regions are in dark grey and untranslated transcription regulator regions are light grey. The red arrows indicate sites of T-DNA insertions for *ndc1* and *gp210* homozygous knockout lines.

# Chapter 4

# Knockout Line Phenotyping and Fasciations

## 4.1 Introduction

In *Arabidopsis* and other species, the analysis of the growth and development of a knockout line is integral to understanding the function of the disrupted gene. Differences in the growth and development of mutant lines with loss-of-function compared to wild-type lines can suggest what roles the protein encoded by the gene may play within a cell (Bolle et al. 2011; Bouché and Bouchez 2001).

Reverse (gene to phenotype) genetic approaches have been used in deciphering gene functions in *Arabidopsis.* Due to the increasing knowledge of the *Arabidopsis* genome, reverse genetic screens have become possible by studying the phenotypes of plant lines with known gene inactivations. Reverse genetic approaches have targeted numerous plant Nup homologs, and analysis has demonstrated highly pleiotropic phenotypes (Xu and Meier 2008). These variations can be summarised as changes in germination, growth, flowering, and seed set, and are discussed separately in the following sections.

### 4.1.1 Plant Nups and plant growth

A diverse range of developmental defects have been observed in *Arabidopsis* Nup knockout mutants. These include stunted growth, impaired stamen development, altered juvenile/adult transition and altered phyllotaxy (reviewed in Meier and Brkljacic 2009). Pleiotropic developmental defects are seen in the mutants *sar1*, *sar3*, and *attpr*, which are mutations of plant NPC structural Nups SAR1, SAR3, and AtTpr that contain FG-repeats and are involved in mediating nuclear transport These developmental defects include smaller plant size, abnormal phyllotaxy, and terminal flower structures (Dong et al. 2006; Jacob et al. 2007; Parry et al. 2006; Xu et al. 2007; Zhang and Li 2005).

The size, shape, and number of leaves can also be significantly reduced in *Arabidopsis* Nup mutants, and in mutants of nuclear transport regulators. Fewer leaves were observed at bolting in *Arabidopsis* T-DNA mutant *nup136*, a mutant of FG-repeat containing Nup, Nup136, which is unique to higher plants (Tamura et al. 2010). Mutations in HASTY, an RNA exporter, also cause defects in plant morphology, rolling of leaf blades, smaller leaf size, and the reduction in the number of leaves (Bollman et al. 2003).

### 4.1.2 Plant Nups and flowering

A link between nuclear pore function and flowering-time regulation has been demonstrated with knockouts of many Nup genes (*sar3, sar1, attpr, los4, esd4, nup136)*, causing *Arabidopsis* plants to flower early (Tamura et al. 2010; Xu et al. 2007). T-DNA Nup mutant lines *sar1* and *sar3* displayed irregular spacing of flowers along the stem, and this phenotype was accentuated in crossed double mutants *sar1 sar3*. The early flowering phenotype in Nup mutants *attpr* and *sar3* were observed to be caused by RNA metabolism affecting the floral repressor FLC (Jacob et al. 2007). RNA metabolism is also thought to be involved in the early flowering phenotype displayed by *Arabidopsis* lines deficient in nuclear exportins HASTY and PAUSED (Bollman et al. 2003; Hunter et al. 2003; Li and Chen 2003).

### 4.1.3 Plant Nups and seed set

*Arabidopsis* Nup mutants *nup133* and *nup85* (Nup constituents of the NPC 107-160 sub-complex) have reduced seed production (Xu and Meier 2008). Stunted fruits and fewer mature seeds were produced from a T-DNA insertional mutant of Nup *nup136* (Tamura et al. 2010).

The knockout line *gp210* is annotated as embryo defective in the TAIR database (http://arabidopsis.org/servlets/TairObject?id=132136&type=locus, accessed June 19$^{th}$ 2013). Embryo lethality can be determined from observing in plant siliques. Observation of *ndc1* and *gp210* knockout siliques compared to wild-type for seed yield, size and shape can determine if anchoring-Nups function in seed production.

### 4.1.4 Plant Nups and nuclear morphology

The knockdown of plant Nups in *Arabidopsis* cause difference in cell structure and dynamics (Tamura et al. 2010). Nup136 is a Nup that contains FG-repeats, which are mediators of nucleocytoplasmic transport. Deficiency of Nup136 in *nup136* T-DNA mutants led to the

alteration of nuclear morphology of mutant cells, suggesting Nup136 has a role in the structural maintenance of the nucleus (Tamura et al. 2010).

### 4.1.4 Objectives

Investigating the growth and development of the *ndc1* and *gp210* knockout lines that contain T-DNA insert gene disruptions within the gene homologs will help to determine the role that these genes play and whether they are a part of the NPC. Growth and development measurements standard to studying *Arabidopsis* and, in particular, other Nup-related knockouts, were undertaken. These included analyses of seed germination, leaf number and days before each stage of growth, and root growth. Observations of developmental traits such as coloration and shape of the leaves, stem, and flowers were also conducted to aid in phenotypical analysis. Crosses between the two knockout lines to generate the *ndc1 gp210* double knockout tested their functional redundancy within the complex, and if there is an accentuated effect of mutant phenotype.

## 4.2 Results

### 4.2.1 Plant development and growth

The plant lines selected for the initial experiments in plant growth and development were T65 (*ndc1*) and T83 *(gp210*). Homozygous insertional knockouts for other lines (T74 and T79) could not be isolated, and double KO lines were not yet available when these experiments were conducted.

The bolting stage of *Arabidopsis* plant growth was investigated. This was performed by recording the days before the plant started to bolt and the number of leaves the plant exhibited at bolting, under two growth conditions of 24 h light and 10 h light / 14 h dark cycle. No significant differences were observed in the days before plant bolting of both knockout lines in comparison to the wild-type line (Figure 4.1). The number of rosette leaves recorded at bolting (Figure 4.2) revealed that the *ndc1* knockout line displayed a significant increase (P vaule <0.05) in leaf number under the 10 h light / 14 h dark cycle, compared to the wild-type control. There was no significant difference of the *ndc1* line in 24 h light, nor was there a significant difference for *gp210* knockout line in comparison to wild-type in both growth conditions.

The recorded days before the flowering stage of knockout lines also displayed no significant difference from knockout lines compared to wild-type under both growth conditions (Figure 4.3). The rosette leaf number at flowering (Figure 4.4) showed a small but significant decrease (P value <0.05) of *ndc1* leaves at flowering under 24 h light compared to wild-type. No other significant result in leaf number at flowering was observed for *ndc1* in the 10 h light / 14 h dark cycle and for *gp210* in both growth conditions. The height of the inflorescence stem was also measured at the beginning of flowering to find any other difference in plant structure during the flowering stage. There were no significant differences observed for the stem height at flowering between the knockout lines and the wild-type lines in both growth conditions (Figure 4.5).

Root growth was investigated for the knockout lines by imaging 5 day old seedlings and recording root elongation growth over an additional 48 h. Root elongation measurements (Figure 4.6) revealed a small but significant decrease (P value <0.01) in root growth of the *ndc1* knockout line compared with wild-type. No significant difference was observed for *gp210* roots.

While observing the multiple *Arabidopsis* lines over several weeks during the plant life cycle, there was no consistently replicated difference in plant structure, such as the size and shape of leaves, flowers, and stems, of the knockout lines in comparison to the wild-type controls. After 24 d in 24 h light, plant size and structure were consistently similar for all three lines (Figure 4.7).

In conclusion, the knockout of either *NDC1* or *GP210* seemed to generate no major changes in plant growth and development, at least under the growth conditions tested.

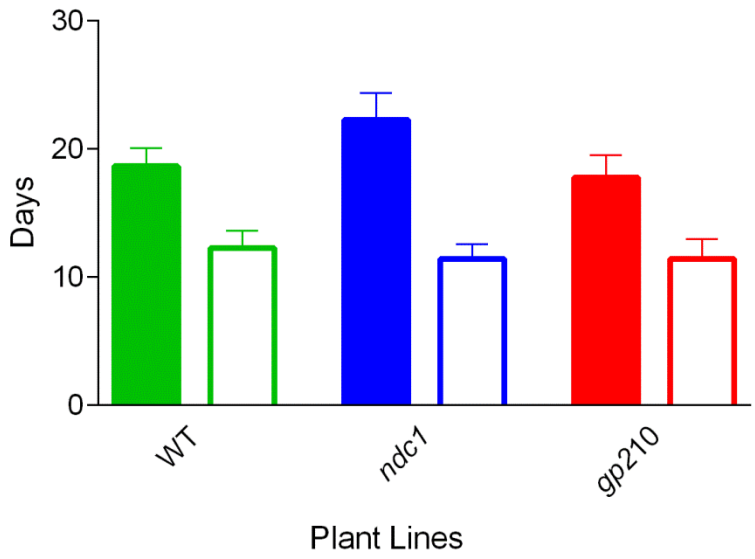


**Figure. 4.1.** Days before plant bolting for different plant lines. Colour filled bars indicate 10 h light / 14 h dark cycle, white filled bars indicate 24 h light regime for the plant line with the corresponding colour. No significant differences were observed between plant lines under the same light regimes. Data are mean values ± SEM, n = 11.

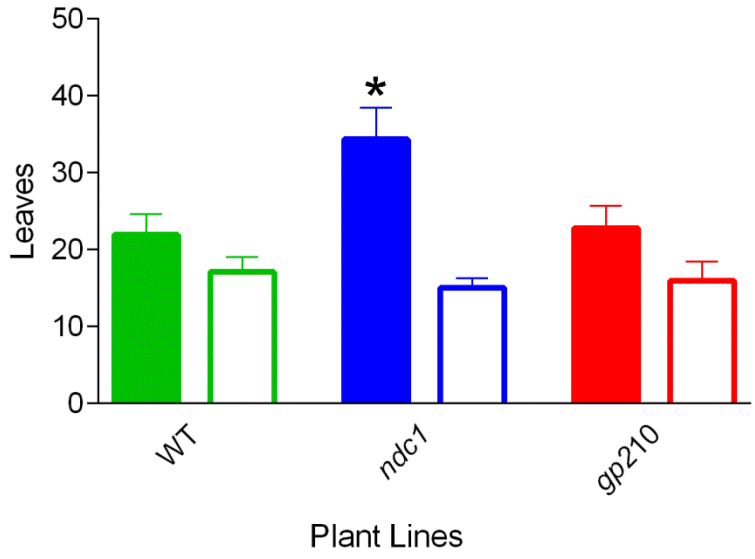


**Figure. 4.2.** Rosette leaf number at bolting for different plant lines. Colour filled bars indicate 10 h light / 14 h dark cycle, white filled bars with coloured borders indicate 24 h light regime for the plant line with the corresponding colour. Knockout line *ndc1* displayed a significant increase in leaf number at the bolting stage under 10 h light / 14 h dark cycle. Data are mean values ± SEM, n = 11. * indicates a significant difference compared to wild-type (WT), P value <0.05.

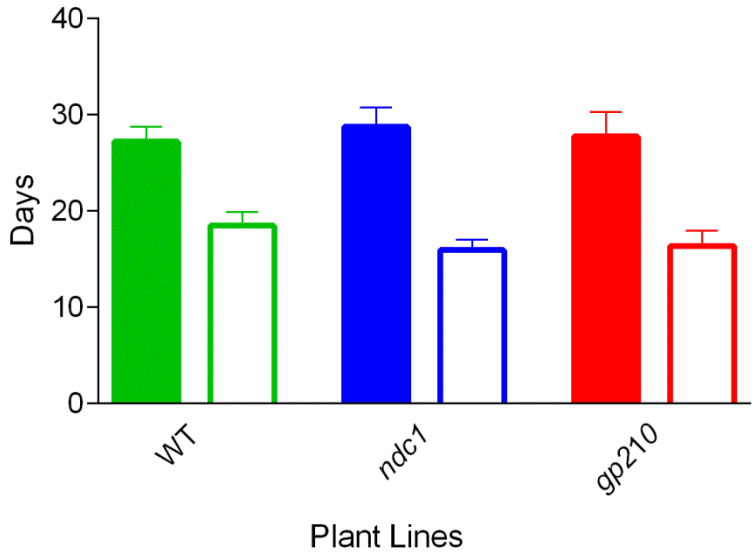


**Figure. 4.3.** Days before flowering for different plant lines. Colour filled bars indicate 10 h light / 14 h dark cycle, white filled bars with coloured borders indicate 24 h light regime for the plant line with the corresponding colour. No significant differences were observed between plant lines observed. Data are mean values ± SEM, n = 11.

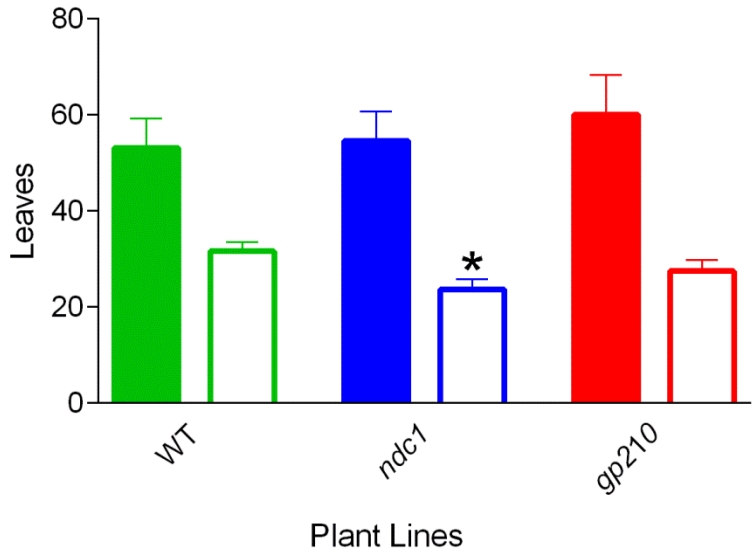


**Figure. 4.4.** Rosette leaf number at flowering for different plant lines. Colour filled bars indicate 10 h light / 14 h dark cycle, white filled bars with coloured borders indicate 24 h light regime for the plant line with the corresponding colour. Knockout line *ndc1* showed a significant reduction of leaf number at the flowering stage under 24 h light. Data are mean values ± SEM, n = 11. * indicates a significant difference compared to wild-type (WT), P value <0.05..

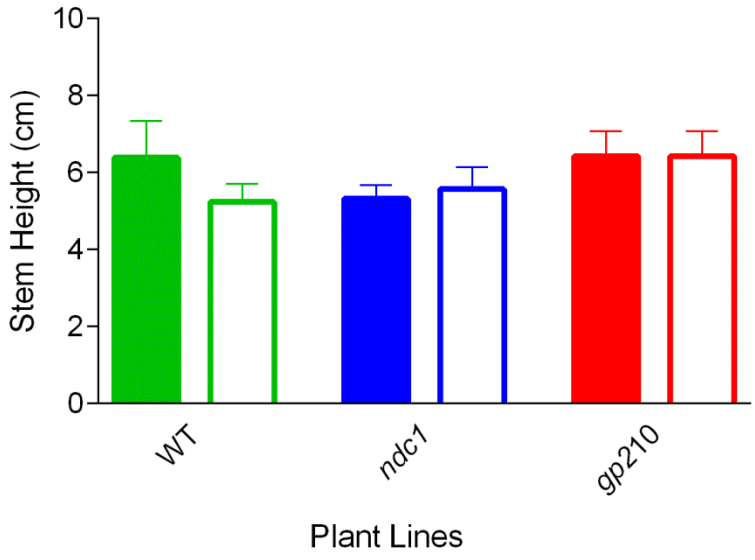


**Figure. 4.5.** Height of stem at flowering for different plant lines. Colour filled bars indicate 10 h light / 14 h dark cycle, white filled bars with coloured borders indicate 24 h light regime for the plant line with the corresponding colour. No significant difference were observed between plant lines observed. Data are mean values ± SEM, n = 11.

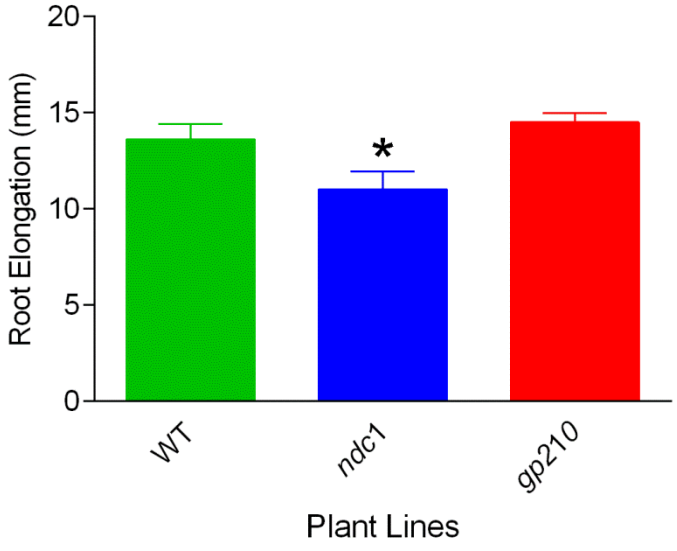


**Figure. 4.6.** *Arabidopsis* 5 day old seedlings were grown for a further 48 h, and elongation during this time was measured. Knockout line *ndc1* displayed a small but significant decrease in root growth. Data are mean values ± SEM, n = 16. * indicates a significant difference compared to wild-type (WT), P value <0.01.

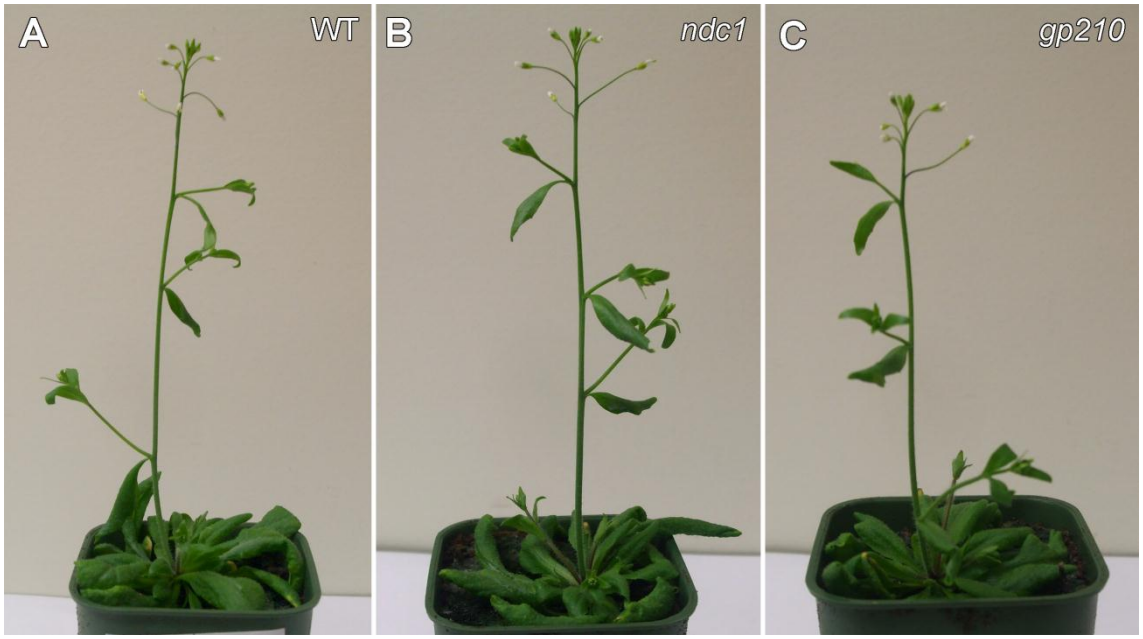


**Figure 4.7.** The growth of the 3 main plant lines under 24 h light after 24 days. There were no observable changes in phenotype.

**A** Wild-type (WT).

**B** The *ndc1* knockout line.

**C** The *gp210* knockout line.

### 4.2.2 Fasciation phenotype

During the initial screening process of the knockout lines, a peculiar phenotype was observed with some plants from the TAIR-supplied seed stock exhibiting wide, flattened stems (Figure 4.8). This phenotype is known as fasciation (Gorter 1965). Plants that exhibited stem fasciation had been grown under 24 h light, albeit with some interruption. As the plants came from the seed stock of plant lines for Nup knockouts T65, T74, T79, and T83, the plants exhibiting fasciation were referred as FT65, FT74, FT79, and FT 83 (see details in Table 2.1). The width of these flattened stem was considerably wider than regular-shaped stems which were circular. There were also a larger number of vascular bundles in the fasciated stem (Figure 4.9), presumably to support the increase in tissue. The inflorescences in a fasciated plant also showed the witches broom appearance typical of fasciated plants, with the number of pedicels being greatly increased and with these being more closely spaced (Figure 4.8D)(Gorter 1965).

There was great interest in further investigating this fasciation phenotype as none of the numerous other plant lines growing under the same growth room conditions displayed the phenotype. These lines included wild-type plants, kinesin motor T-DNA knockout lines, and various GFP expressing lines. However, it was only the four lines from the seed stock of T-DNA Nup knockout lines that exhibited fasciation.

Fasciation is a term used to describe a wide variety of developmental abnormalities in the shoot system (Leyser and Furner 1992). Fasciations have been reported to occur naturally in trees, shrubs, flowers and cacti in at least 107 plant families (Binggeli 1990). Apart from the abnormal shape of the growing apex, fasciations are characterised by an increase in volume and weight of the tissue over a normal plant and by the altered control of growth. However, no new quantitatively different tissues are formed (Gorter 1965). Fasciation phenotypes have been targeted in breeding programs for commercially important species such as tomato cultivars, where an increase in locule number and fruit size was due to fasciation (Tanksley 2004). Fasciations can also be caused by bacterial infections. *Rhodococcus fascians* is a gram positive phytopathogenic bacterial species where infection of dicotyledonous plants can cause fasciations (Crespi et al. 1992).

As a study investigating *R. fascians* was being conducted in the same laboratory as this study, PCR screening of fasciated samples using *R. fascians* specific primers was performed to

determine if phytobacterial infection was the cause of the observed fasciations. Subsequent T-DNA genotyping of fasciated plants and extensive growth studies were also performed to determine if the knockout of *NDC1* and *GP210* was involved in the expression of this structural phenotype.

For investigating the possible contamination of fasciated plants by the bacterium, *R. fascians* specific primers for *R. fascians* were obtained (Table 2.2). PCR screening for the bacterium was performed using the DNA samples of the plants with fasciations. The screening found that *R. fascians* was not present on any plant tissue exhibiting fasciations (Figure 4.10).

The PCR genotyping of the four fasciated plants showed that only two contained confirmed T-DNA knockout inserts (Figure 4.11). Those two plant lines were confirmed as homozygous *ndc1* and *gp210* knockouts. Of the remaining two plants, FT79 revealed a wild-type genotype, while the FT74 plant failed to produce any bands (wild-type or insert) even after multiple DNA extractions and PCR tests.

To confirm any link between T-DNA insertions and the fasciation phenotype, PCR screening was conducted using T-DNA specific primers that amplify DNA within the T-DNA insertion. Primers were designed for within the T-DNA vector sequences of SAIL and SALK (Table 2.2). Four primer pairs (two each for SAIL and SALK) were designed to produce PCR product fragments of particular length (see Table 2.2). Positive T-DNA SAIL and SALK controls were used in testing the primers, and all four of the primer pair sets produced the expected PCR product DNA fragment lengths (Figure 4.12). These primers could then be used in T-DNA PCR screening of fasciated plants.

Primer sets SAIL-914 and SALK-1452 were selected for T-DNA insert screening of fasciated plants, since they produced clear gel bands in primer tests and could be distinguished from each other by size. The screening for the SAIL T-DNA insert confirmed the presence of the insert in the FT65 plant, and that there was no SAIL insert in lines FT74, FT79, and FT83 plants (Figure 4.13). The SALK T-DNA screening confirmed the presence of the insert in plant FT83. This insert was also found in plant FT74. However, plants FT65 and FT79 did not contain the SALK insert (Figure 4.14). Although FT74 did not produce a product from the specific T74 primer PCR reaction, the T-DNA SALK screening determined there was an insert present, at an unknown location. Since FT79 was not found to have an insert present, no connection between fasciation and T-DNA Nup gene disruption could be confirmed.

Numerous attempts were also made to replicate the phenotype from the progeny of the fasciated plants. Seeds were harvested from the four plants and planted along with wild-type plants. These plants were subjected to the same growth settings of 24 h light, as well as to a range of other growth conditions including the 10 h light 14 h dark cycle. However, the fasciated phenotype was not replicated in any of the progeny or control plants.

The primary difficulty in replicating the fasciation is that the plants exhibiting fasciations were grown during the period of the February 2011 Christchurch earthquake when growth rooms were subject to continued power losses and temperature fluctuation. Further, these plants were subject to continuous pruning to retain the influoresences within the plastic cover sleeves. There can be numerous causes for plant fasciation including insect attack, mechanical pressure and/or tension, time and density sowing, temperature fluctuation, mineral deficiency and biotic stresses (Gorter 1965; Iliev and Kitin 2011). It is suggested, therefore, that the cause of fasciation phenotype may be unusual growth stresses on these plants. However, why these plants and no others within the growth room should have developed fasciation under these conditions is unclear.

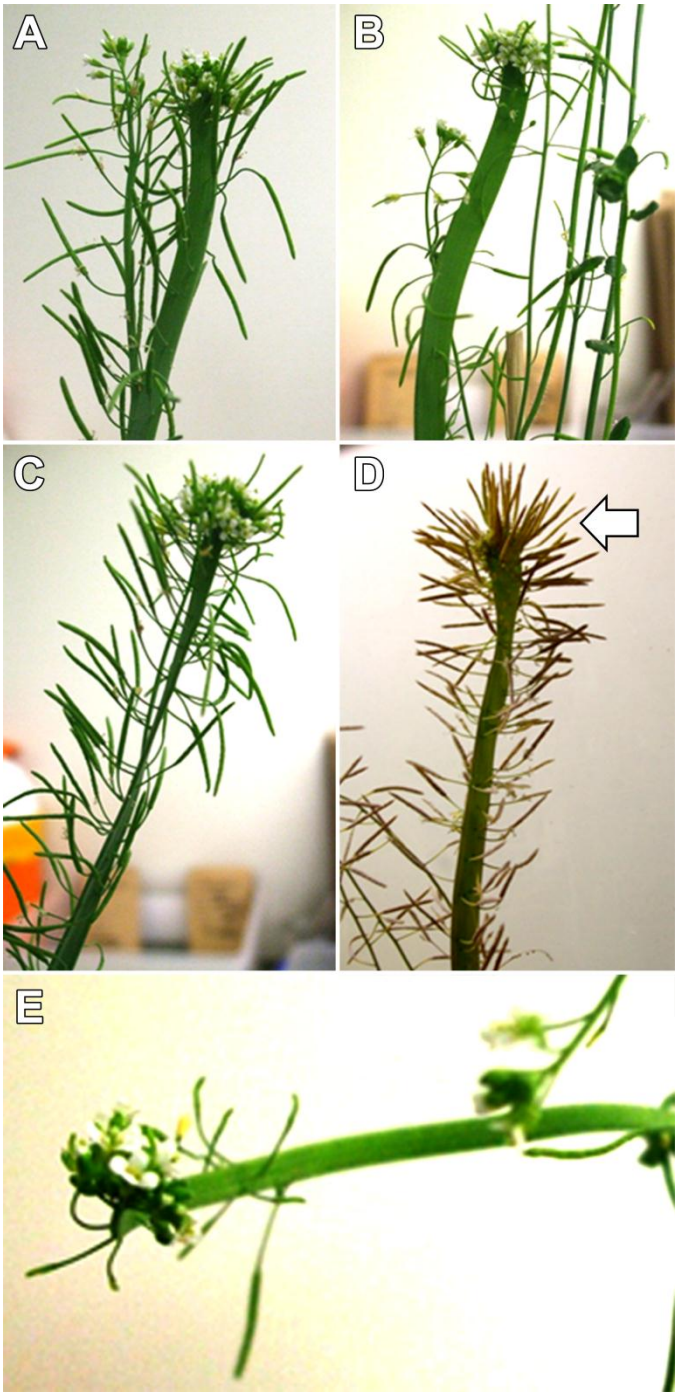


**Figure 4.8.** Photos of *Arabidopsis* plants exhibiting stem fasciations. Plants were under 24 h light conditions and grown during the Christchurch earthquake period. Arrow indicates witches broom at inflorescence.

**A** FT79 after 12 weeks.

**B** FT83 (*gp210* confirmed) after 12 weeks.

**C** FT79 after 12 weeks.

**D** FT79 after 17 weeks.

**E** FT65 (*ndc1* confirmed) after 12 weeks

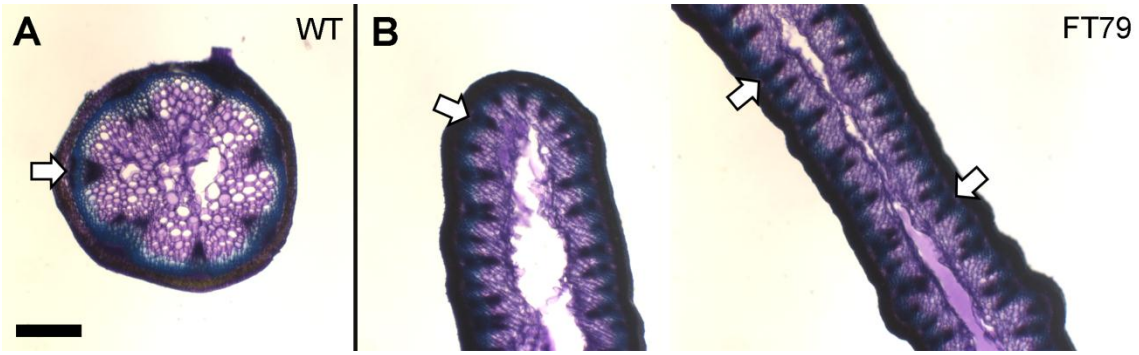


**Figure 4.9.** Cross sections of wild-type (WT) and fasciated stem stained with 0.1% toluidine blue. Arrows indicate vascular bundles. Scale bar = 500 µm.

**A** Wild-type (WT) stem.

**B** Fasciated stem from plant FT79.

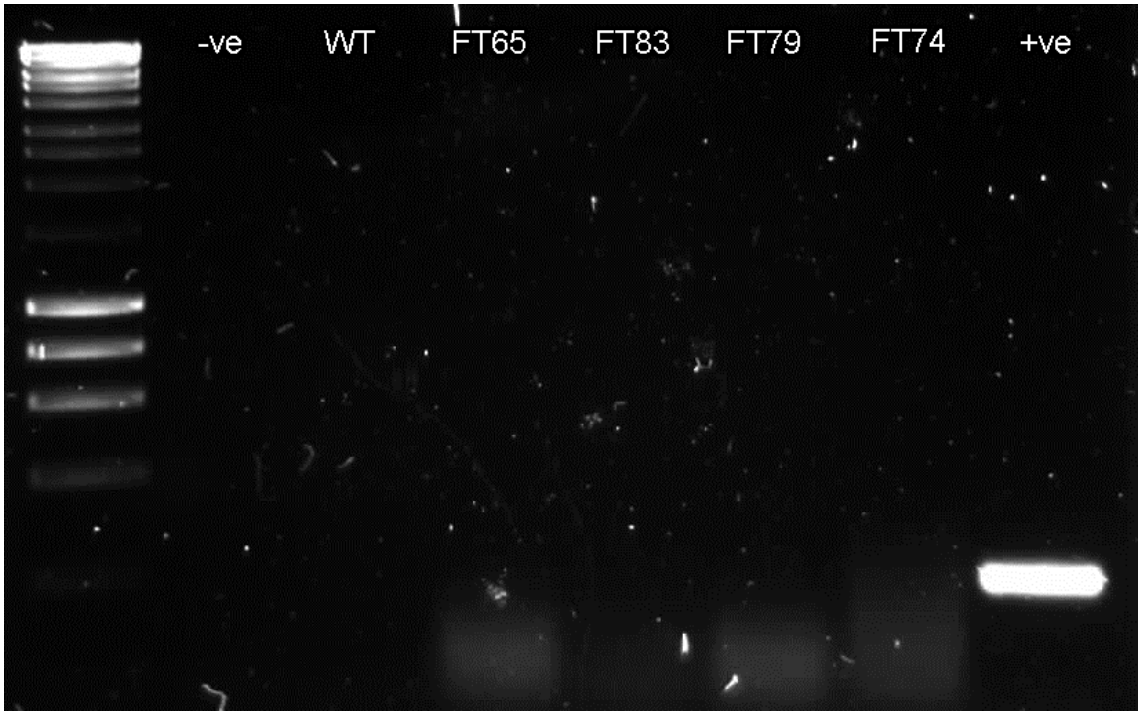


**Figure 4.10.** PCR screening of plants exhibiting fasciations. Plants FT65, FT83, FT79 and FT79 were checked for infection with *Rhodococcus fascians*. Samples were compared to a negative water control (-ve), wild-type plants (WT) and a positive control (+ve) from an *R. fascians* colony. Only the positive control sample showed a 210 bp fragment, indicating the absence of *R. fascians* contamination in the fasciated plants.

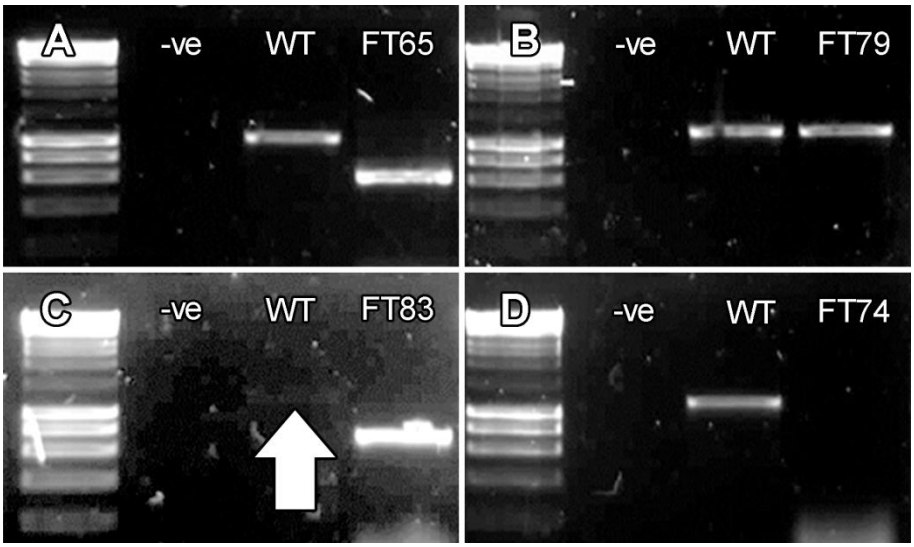


**Figure 4.11.** Genotyping of fasciated plants FT65, FT83, FT74, and FT79.

The arrow indicates faint wild-type (WT) T83 band. Negative water control (-ve). Only plants FT65 and FT83 were confirmed as T-DNA knockouts. The plant FT79 displayed a wild-type band while FT74 plant produced no band.

PCR primer conditions for every three lanes:

**A** T65 genomic primers + SAIL insert primer for plant FT65.

**B** T79 genomic primers + SALK insert primer for plant FT79.

**C** T83 genomic primers + SALK insert primer for plant FT83.

**D** T74 genomic primers + SALK insert primer for plant FT74.

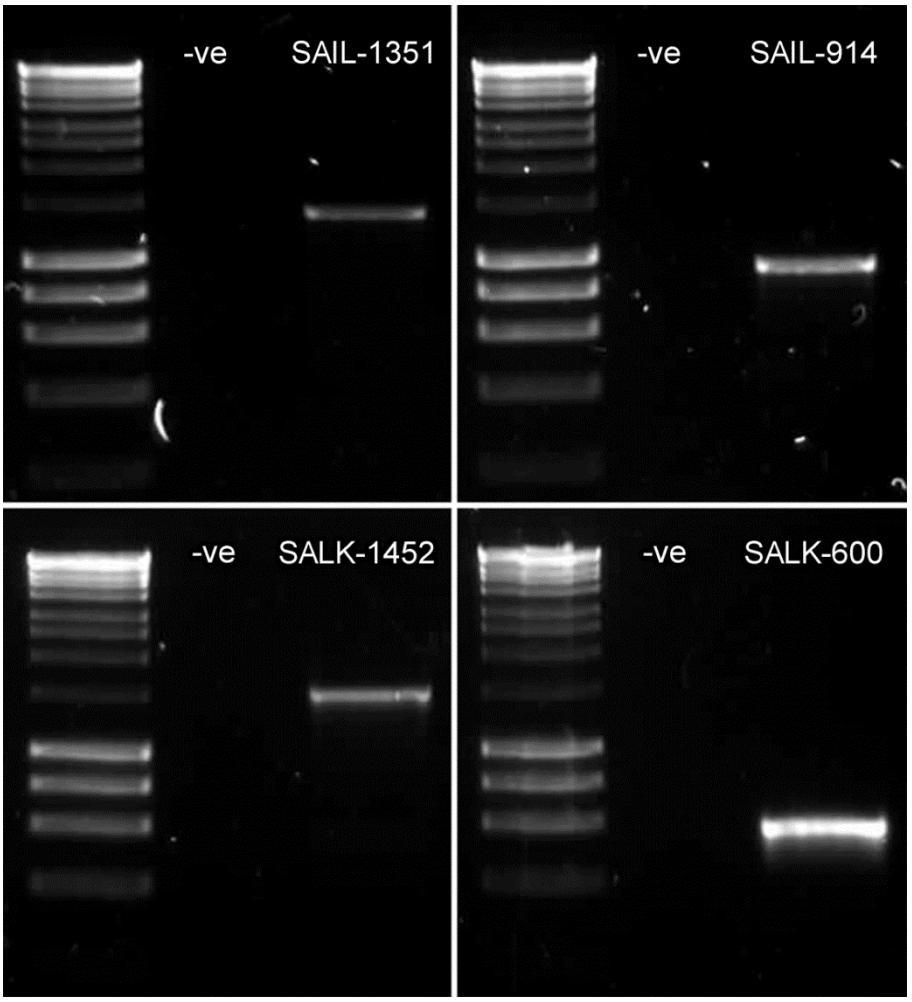


**Figure 4.12.** T-DNA insert primer tests for screening fasciated plants containing SALK or SAIL T-DNA insertions. Numbers indicate the amplified DNA fragment length (bp) from within the insertion. Negative water control (-ve), Positive SAIL controls: *ndc1* knockout sample, Positive SALK controls: *gp210* knockout sample.

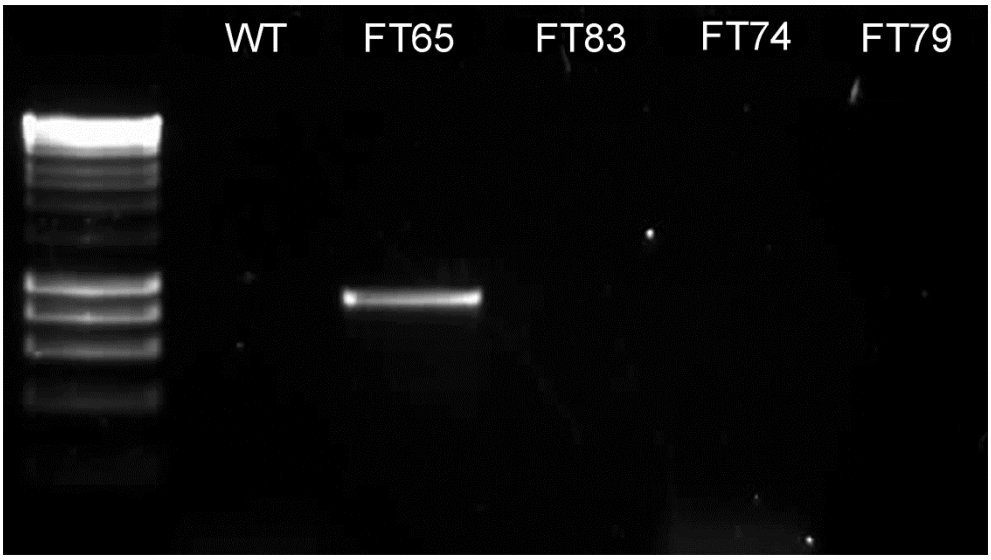


**Figure 4.13.** SAIL-914 T-DNA insert PCR screening of fasciated plants from plants FT65, FT83, FT74, and FT79. Only FT65 shows 914 bp fragment, indicating the presence of the SAIL T-DNA insert.

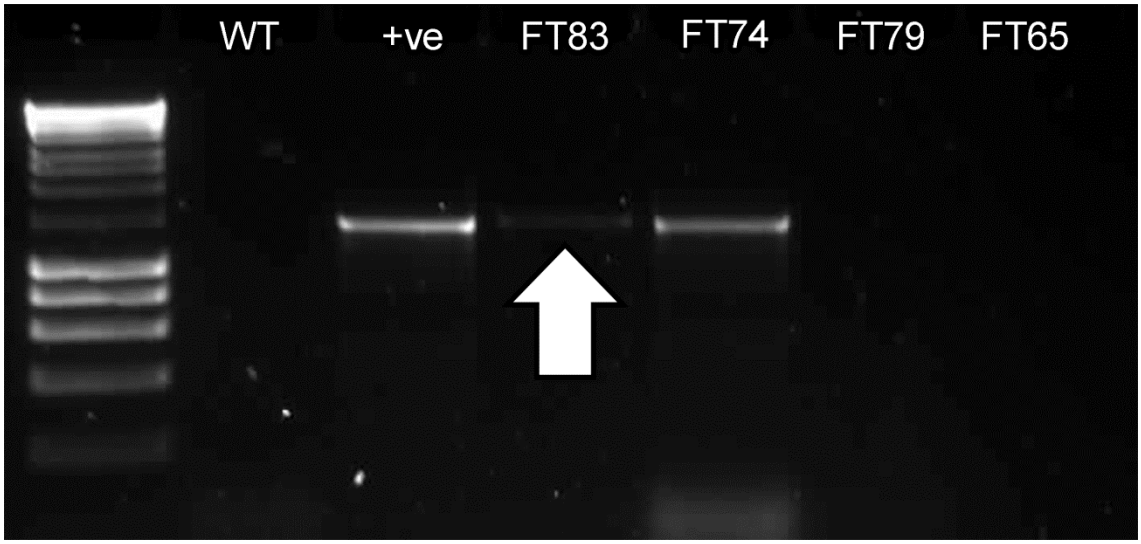


**Figure 4.14.** SALK-1452 T-DNA insert PCR screening of fasciated plants from lines: FT65, FT83, FT74, FT79. Along with the *gp210* knockout sample positive control (+ve), plants FT83 and FT74 show a 1452 bp fragment, indicating the presence of SALK T-DNA insert, although for T83 this band (arrow) was faint.

### 4.2.3 Double knockout crosses

Because there were only minimal differences in the growth and development of *ndc1* and *gp210* knockout lines compared to wild-type, crosses between these lines were performed to create the double knockout *gp210 ndc1* to determine if there would be a synergistic effect from the disruption of two anchoring-Nup proteins. Crosses were also conducted with nuclear GFP expressing lines to image the nuclear morphology of knockout lines.

The *Arabidopsis* line CS84731 (T11), which expresses GFP targeted to the nucleus (Cutler et al. 2000), was crossed with *gp210* (Figure 4.15A). The GFP line was used as the male parent to pollinate the ovules of *gp210* female flowers. The F1 seed progeny was planted on agar growth plates and, after 5 days, screened with a stereofluoresence microscope for nuclear GFP to confirm the successful cross. Confirmed nuclear GFP *gp210* plants were grown and selfed, and the F2 progeny was harvested and grown for PCR genotyping. F2 plants were screened with PCR to isolate homozygous *gp210* knockouts that expressed nuclear GFP. F2 plants were also screened for homozygous nuclear GFP. The progeny of selfed F2 lines were screened with a stereofluoresence microscope, and an F2 plant producing progeny that all express nuclear GFP was homozygous for this transgene. This nuclear GFP *gp210* line was then used to pollinate a homozygous *ndc1* plant to create a double heterozygous *gp210 ndc1* line (Figure 4.15B). F1 progeny were screened with the stereofluoresence microscope for nuclear GFP, grown and then selfed.

The F2 generation were screened by PCR and fluorescence to isolate the double *gp210 ndc1* genotype. The expected genotype frequency ratios of F2 generation would be similar to that of a dihybrid cross (Table 4.1), and any divergence from this expected frequency would indicate changes in the viability of the double knockout progeny. A total of 105 plants from the F2 generation were screened with PCR primers for both inserts to determine genotype frequencies and also to isolate double *gp210 ndc1* homozygotes.

The observed distribution of genotypes in F2 double knockout *gp210 ndc1* plants was different to the expected genotype frequencies (Table 4.2). Chi-square analysis confirmed a significant difference (P value $0.0273<0.05$) of the observed genotypes from the expected frequencies (Table 4.4). The distribution was observed to have less plants possessing both the heterozygous and homozygous *ndc1* genotypes. The knockout genes *ndc1* and *gp210* from the F2 progeny were separated and confirmed the difference in observed frequencies to expected

was from the *ndc1* knockout distribution (Table 4.3). Expected punnet ratios of single knockout lines are 1:2:1: *GP210* displays that distribution ratio (P value 0.8546>0.05). However *NDC1* displays a 2:2:1 ratio (P value 0.0002<0.0001) (Table 4.3). No interaction between the two knockouts was observed in chi-square analysis on a single knockout genotype against the other knockout genotype frequencies. Significant P values (< 0.05) were only observed in analyses involving homozygous *ndc1* (Table 4.4). These analyses indicate that homozygous *ndc1* genotype was unfavourable among the F2 progeny. It is possible that embryo or seed abortion is involved.

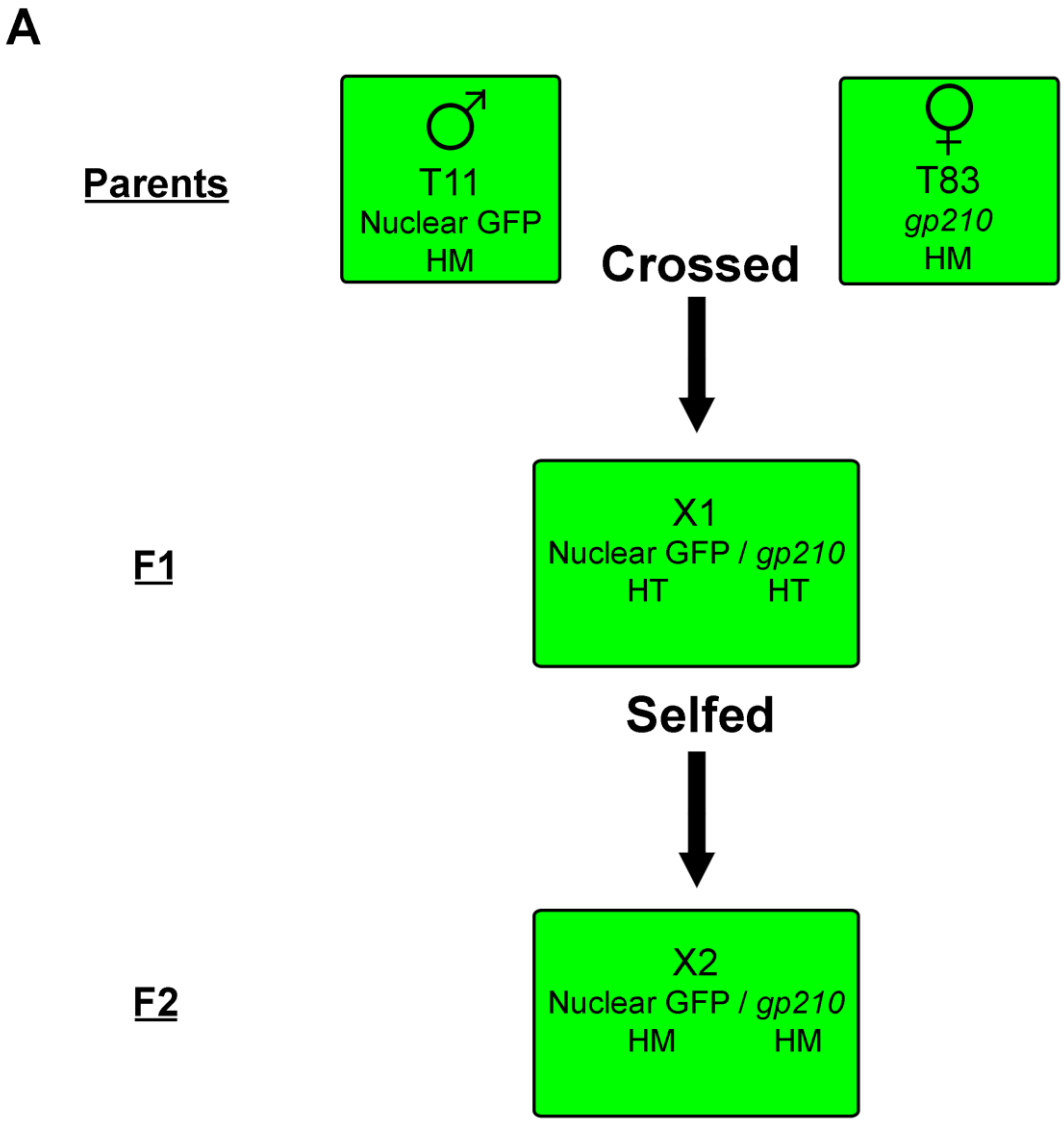


**Figure 4.15.** Diagrammatic representation of the crossing strategy used to generate double knockout.

**A** The cross between a nuclear GFP line and *gp210*, and subsequent F1 and F2 generations. Progeny of the F2 generation was screened for nuclear GFP homozygosity.

WT = Wild-type.

HT = Heterozygous.

HM = Homozygous.

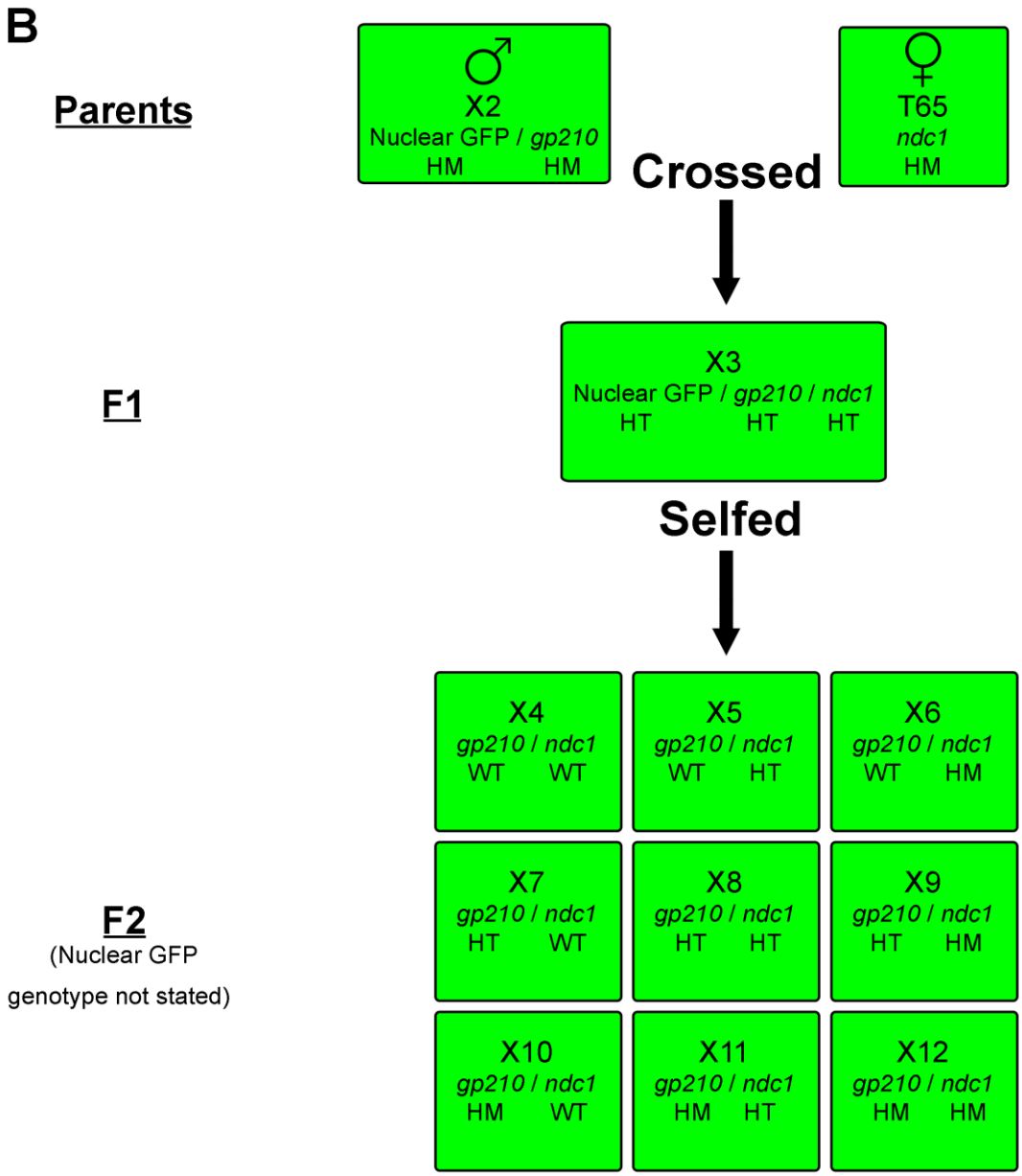


**Figure 4.15. (continued)**

**B** Diagram of the cross between *gp210* and *ndc1*, and subsequent F1 and F2 generations. Nuclear GFP ignored for F2 to focus on double knockout gene. The F2 progeny generation shows all possible genotypes for screening.

WT = Wild-type.

HT = Heterozygous.

HM = Homozygous.

**Table 4.1.** T-DNA dihybrid expected knockout genotype distribution frequencies of X3 progeny (double knockout *gp210 ndc1* heterozygotes). WT = Wild-type, HT = Heterozygous, HM = Homozygous.

| | | *gp210* | | |
|---|---|---|---|---|
| | | WT | HT | HM |
| *ndc1* | WT | 1 | 2 | 1 |
| | HT | 2 | 4 | 2 |
| | HM | 1 | 2 | 1 |

**Table 4.2.** Observed and expected knockout genotypes in F2 from selfed X3 (double knockout *gp210 ndc1* heterozygotes). A total of 105 plants were screened. WT = Wild-type, HT = Heterozygous, HM = Homozygous.

| Plant Code | Genotype | | Expected | Observed |
|---|---|---|---|---|
| | *gp210* | *ndc1* | | |
| X4 | WT | WT | 6.6 | 11 |
| X5 | WT | HT | 13.1 | 10 |
| X6 | WT | HM | 6.6 | 3 |
| X7 | HT | WT | 13.1 | 22 |
| X8 | HT | HT | 26.2 | 24 |
| X9 | HT | HM | 13.1 | 9 |
| X10 | HM | WT | 6.6 | 11 |
| X11 | HM | HT | 13.1 | 10 |
| X12 | HM | HM | 6.6 | 5 |
| | Total | | 105 | 105 |

**Table 4.3.** Observed and expected single knockout genotypes in F2 from selfed X3 (double knockout *gp210 ndc1* heterozygote).

| Genotype | Expected | *gp210* | *ndc1* |
|---|---|---|---|
| Wild-type | 26.3 | 24 | 44 |
| Heterozygous | 52.4 | 55 | 44 |
| Homozygous | 26.3 | 26 | 17 |
| Total | 105 | 105 | 105 |

**Table 4.4.** Chi-Square P value Table of F2 dihybrid T-DNA knockout lines.
WT = Wild-type, HT = Heterozygous, HM = Homozygous.
* indicates significance, P value <0.05.

| | | *gp210* | | | P value | Total |
|---|---|---|---|---|---|---|
| | | WT | HT | HM | | |
| *ndc1* | WT | 11 | 22 | 11 | 0.9999 | 44 |
| | HT | 10 | 24 | 10 | 0.8338 | 44 |
| | HM | 3 | 9 | 5 | 0.7674 | 17 |
| | P value | 0.0498* | 0.0297* | 0.1253 | | 0.0002* |
| Total | | 24 | 55 | 26 | 0.8546 | 0.0273* |

### 4.2.4 Observations of knockout line germination and seed set

The germination of knockout lines seed was recorded. As no significant differences were observed in the germination rate of knockout lines compared to wild-type in a single experiment (Table 4.5), there was no evidence to justify further investigation needed of seed germination.

The seed set in siliques of knockout lines were imaged from a stereomicroscope to observe possible aborted seeds or embryo abortion. Aborted embryos can be visualised as gaps within the silique pod. Seeds that have been aborted look misshapen and darker in colour (http://www.seedgenes.org/Tutorial.html). The knockout line *gp210* was imaged due to being annotated as embryo lethal on the TAIR database. (http://arabidopsis.org/servlets/TairObject?id=132136&type=locus, accessed June 19th 2013).

The seed set of *gp210* knockout line and double knockout line *gp210 ndc1* displayed no differences in seed set compared with wild-type lines (Figure 4.16). No aborted embryos of seed were observed. The shape, size and colour of knockout line seeds in silique pods were observed to be similar to wild-type.

**Table 4.5.** Seed germination of *Arabidopsis* lines.

| Code | Germinated seeds | Total | Germination % |
|---|---|---|---|
| WT | 149 | 154 | 96.8% |
| *gp210* | 129 | 132 | 97.7% |
| *ndc1* | 123 | 127 | 96.9% |
| *gp210 ndc1* | 144 | 148 | 97.3% |

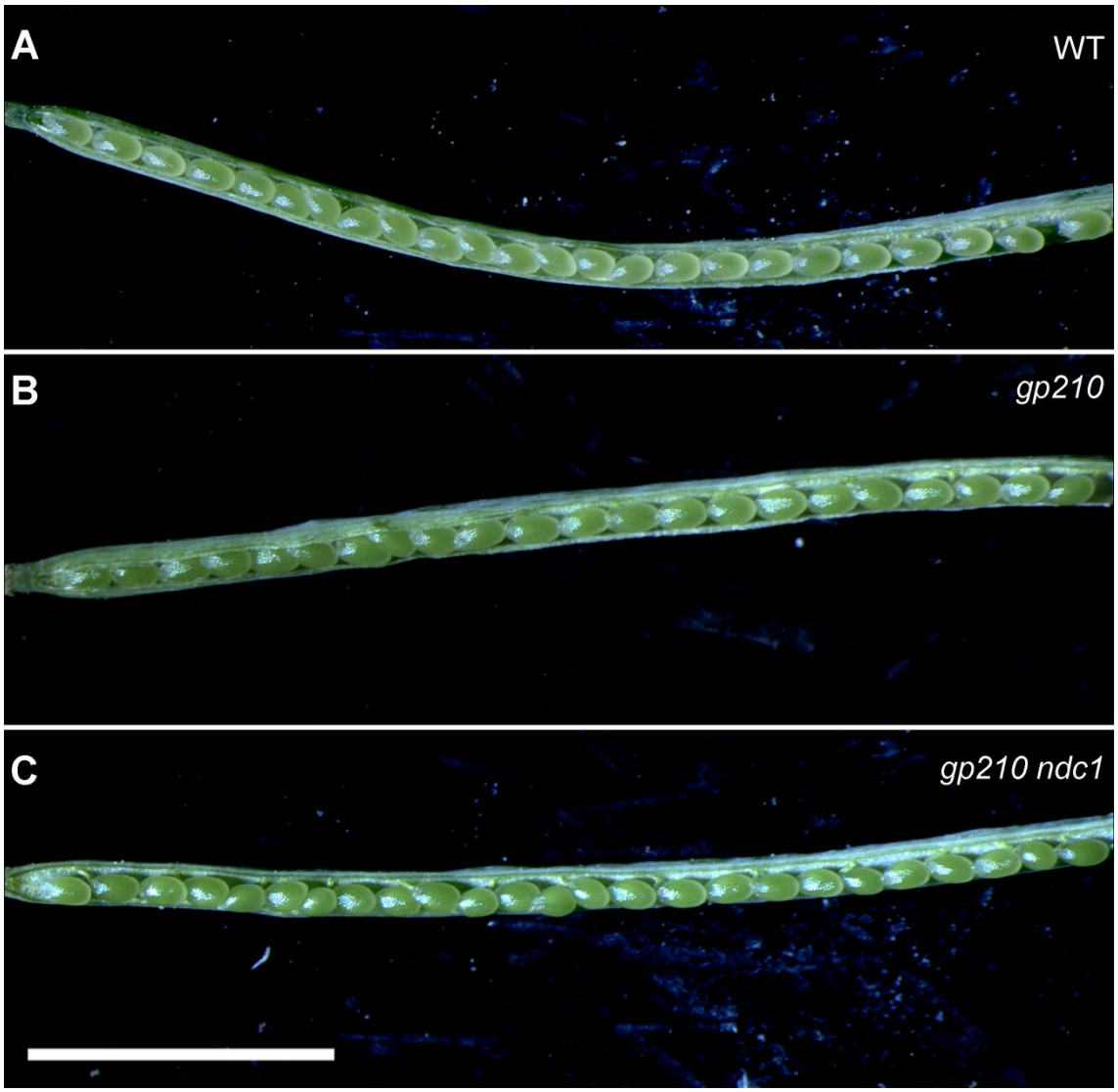


**Figure 4.16.** Silique images of *gp210* knockout and double *gp210 ndc1* knockout. No seed abortions were observed. Scale bar = 5 mm.

### 4.2.4 Nuclear morphology of double knockout lines

The structure of the nucleus in *gp210 ndc1*double knockout line was observed in the cells of 5 days old roots expressing nuclear GFP. Homozygous double knockout *gp210 ndc1* line was selfed, and the seeds were plated onto agar growth plates. After 5 days of growth the seedlings were screened with a stereofluoresence microscope. GFP expressing seedlings were isolated and the root cells and root tip were imaged with confocal microscopy along with nuclear GFP control line (T11) (Figure 4.17). The nuclei of *gp210 ndc1* root cells displayed no difference in size and morphology to the control line. The knockout of both anchoring-Nup homologues does not affect the structure and integrity of the cell nucleus. Therefore, anchoring-Nups may not be involved in the structure of the plant nucleus or that there may be other anchoring-Nup subunits yet to be identified that can replace *NDC1* and *GP210* function.

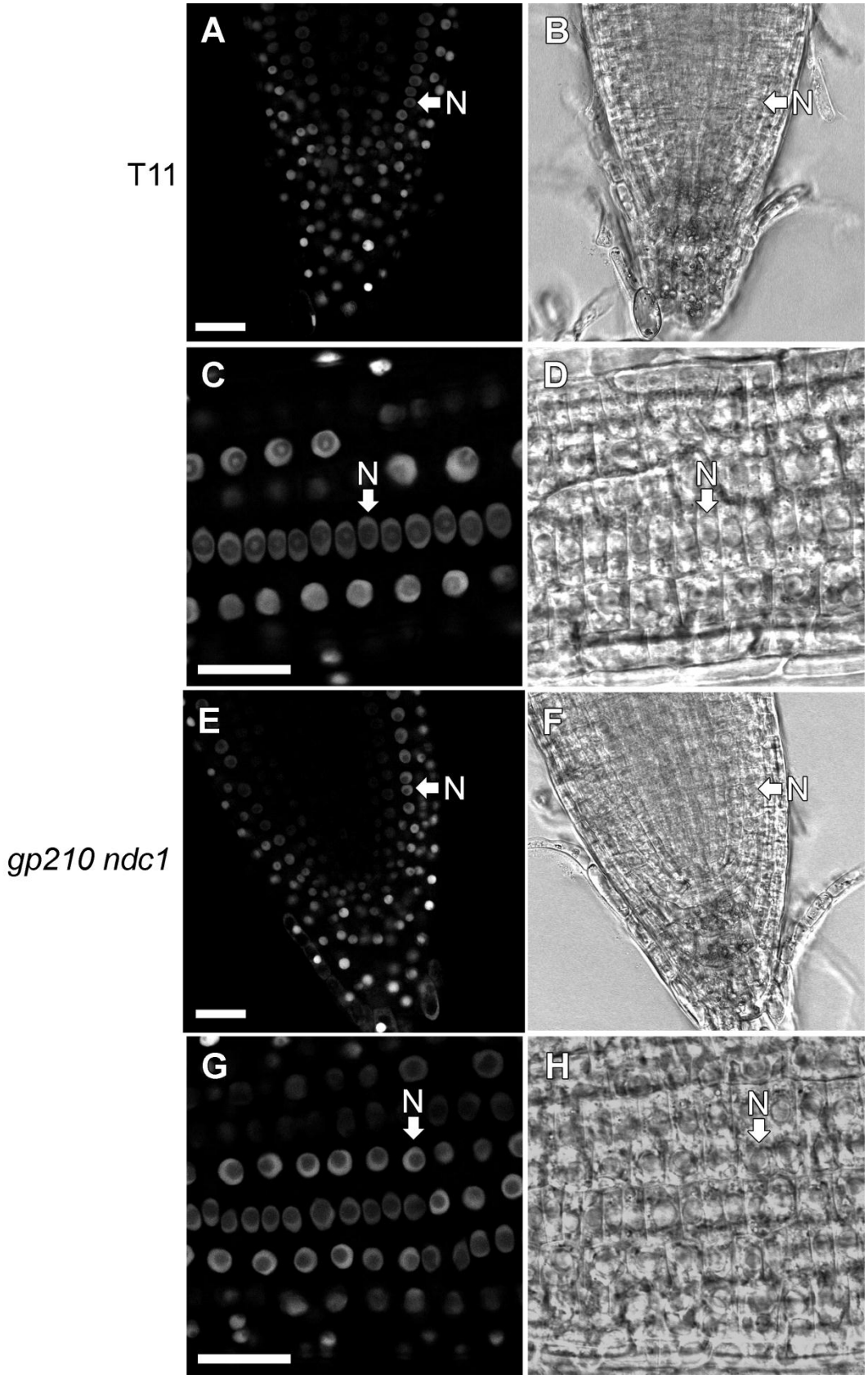


**Fig 4.17.** *Arabidopsis* 5d old roots with cells stably expressing GFP targeted to the nucleus. N marks cell nuclei. *gp210 ndc1* double knockout nuclei does displays similar morphology to control line T11. Scale bars = 25 µm.

## 4.3 Conclusions

Of the two Nup knockout lines, *ndc1* and *gp210,* it was only *ndc1* that displayed significant differences in plant development. The number of leaves for *ndc1* differed from wild-type at the bolting and flowering stages, with significantly more leaves at bolting under 10 h light / 14 h dark cycle and significantly less in 24 h light growth conditions at flowering. This difference in *ndc1* showed that the knockout line exhibited changes in leaf development under different growth conditions and stress. Changes in root growth were also observed in *ndc1* with a significant decrease of root elongation in comparison to wild-type.

Double knockout lines *gp210 ndc1,* created to observe the effect of multiple subunits in the anchoring system being knocked out, showed no differences in seed set or nuclear morphology compared to wild-type and controls. However the PCR genotyping of the F2 progeny from the double heterozygote *gp210 ndc1* line found a significant difference in the genotype frequency distribution for *ndc1* indicating a negative effect toward plants homozygous *ndc1*. There no was synergistic interaction observed between *ndc1* and *gp210* knockouts, since divergence of observed genotype distribution to the expected distribution was only displayed in *ndc1* and not exacerbated by *gp210*.

With the onset of the fasciation phenotype, many attempts were made to find a link between the phenotype and T-DNA insertion knockout of plant anchoring-Nups. After multiple screenings, no connection was found between the phenotype and knockouts. Many attempts were performed to replicate the phenotype using the progeny from the fasciated plants. Fasciation was not however, observed after the initial four plants. The plant exhibiting fasciations were growing under stressful conditions, which included the 2011 Christchurch earthquake, following power outages, and multiple prunings to retain their growth in plastic enclosures. These uncontrolled, stressful conditions were determined to be the most likely the cause of the fasciations in the four plants. Furthermore, of the four fasciated plants, only two could be confirmed on containing a homozygous insert. These were plants FT65 and FT83, and were equivalent to the *ndc1* and *gp210* knockout lines. Of the remaining two plants, the FT74 plant, which was from a seed stock for a *GP210* knockout, showed no wild-type bands (Figure 4.11D). However, the presence of the SALK T-DNA insert band (Figure 4.14) suggests that the *GP210* gene may indeed have been knocked out, although sequencing would be required to confirm this. The fourth fasciated plant, FT79, showed a wild-type band

(Figure 4.11B) and lacked the SALK insert (Figure 4.14), and is likely not an insertional mutant. At some stage, however, the *NDC1* gene from this line should be sequenced to confirm the lack of gene disruption.

# Chapter 5

# Nucleocytoplasmic Transport of Knockout Lines

## 5.1. Introduction

### 5.1.1 *Arabidopsis* root growth assays

*Arabidopsis* will often show hypersensitivity to drugs that target the pathway in which the mutation lies. For example, the temperature-sensitive mutant *microtubule organisation 1* (*mor1-1*) displays hypersensitivity to microtubule depolymerising drug oryzalin at higher temperatures, while the cellulose sensitive mutant *rsw1* shows hypersensitivity to the cellulose synthesis inhibitors dichlobenil and isoxaben (Collings et al. 2006). Similarly, the dynamin mutant *rsw9* is hypersensitive to monensin, an endocytosis inhibitor (Collings et al. 2008). Similar experiments have also been used to characterise *Arabidopsis* hormone-related genes in mutants initially identified through changes in sensitivity to exogenously applied hormones or inhibitors. Examples of this include the growth responses demonstrated in *sax1* dwarf mutant to hormones abscisic acid, auxin, gibberellins, ethylene, and brassinosteroid (Ephritikhine et al. 1999), and protein kinase mutants *snrk2.2* and *snrk2.3* root growth response to hormone abscisic acid (Fujii et al. 2007). Importantly, the use of applied inhibitors can often identify a phenotype which is otherwise hidden.

It was thought therefore that Nup knockout lines *gp210* and *ndc1* might display hypersensitivity when subjected to inhibitors of nuclear transport. Root growth assays of *ndc1* and *gp210* seedlings on nuclear transport specific drugs would display the importance of the genes within the NPC system.

### 5.1.2 Nuclear transport inhibitor leptomycin B

Leptomycin B (LMB), a potent antibiotic, is an important tool in the study of nuclear export. LMB inhibits the active export of most, if not all, macromolecules from the nucleus (Asscher et al. 2001). Nucleocytoplasmic transport through the NPC is mediated by karyopherin proteins. Members of this large protein family include importins and exportins, which facilitate nuclear import and export (Mosammaparast and Pemberton 2004). Proteins that contain the nuclear export signal are dependent on exportin1 for export from the nucleus (Nishi et al. 1994). LMB is a nuclear export inhibitor in animal cells (Komiyama et al. 1985) and fungal cells (Hamamoto et al. 1983) where it targets exportin1 by alkylating the Cys residue of exportin1.

As with animal cells LMB has been used in understanding nucleocytoplasmic dynamics in *Arabidopsis*. The effects of LMB have been visualised in *Arabidopsis* cells expressing RSZp22-GFP, where RSZp22 is a splicing regulator protein, when treated with LMB photobleaching demonstrated that LMB blocked the export mechanism of RSZp22 (Tillemans et al. 2006).

### 5.1.3 Observing nuclear transport using the PUM protein

In studying nuclear transport within *Arabidopsis* lines, a protein known to be transported through the NPC attached to a fluorescent reporter can be used to visualise nucleocytoplasmic transport. This fusion construct would be used as an indicator of nuclear transport and also as a control in nuclear transport inhibition. One class of proteins that travels through the NPC are the PUF family of proteins. These proteins are gene regulators which control gene expression at the post transcriptional level by promoting RNA decay and repressing translation (Wharton and Aggarwal 2006). Pumilio homology domain (PUM) proteins are a conserved group of proteins within the PUF family of proteins that bind specifically to RNA with sequence specificity and which function in RNA decay (Miller and Olivas 2011). When *Arabidopsis* PUM proteins were transiently expressed as GFP or RFP fusions in onion and fava bean epidermal cells, the proteins localised to the cytoplasm and small loci within the nucleus. When treated with LMB, however, the PUM proteins accumulated in the nucleus which indicated that it was common for these proteins to shuttle between the nucleus and cytoplasm

(Tam et al. 2010). The GFP-PUM and RFP-PUM proteins provide an ideal control system to confirm the activity of LMB in Nup protein fluorescent lines.

### 5.1.4 Particle bombardment transformation of *Arabidopsis*

Transient expression has been an indispensable tool for the study of gene function. Nucleocytoplasmic dynamic transport of *gp210* and *ndc1* knockout lines can be observed in transformed cells transiently expressing the PUM protein fused to GFP or RFP. Particle bombardment is a transformation technique that can be used for both transient expression studies and creating stable transformants of various plants (Christou 1994). The technique was first described as a method of gene transfer into plants where tungsten particles carrying DNA or RNA were accelerated to pierce the cell walls and membranes and enter intact onion epidermal cells without killing them, with the protein subsequently being expressed (Klein et al. 1987).

Transformation by particle bombardment has previously been conducted in *Arabidopsis*, in cultured root sections, transgenic plants expressing introduced DNA (neomycin phosphotransferase II) were regenerated from callus tissue after incubation (Seki et al. 1991). Other methods of particle bombardment in *Arabidopsis* include particle delivery into the epidermis of *Arabidopsis* leaves (Ueki et al. 2009). These techniques have successfully yielded transgenic cell lines, but require several weeks for the preparation of cultured samples and need plant growth of leaves before particle bombardment transformation can be performed. As the size and shape of *Arabidopsis* callus and leaf cells are not optimal for observing the effects of LMB, a new method was developed in this study to deliver foreign DNA by particle bombardment into the root cells of 10 day old *Arabidopsis* seedlings. The aim of this method was to create transiently expressed root cells within two weeks from initial seed planting, and to visualise clear nucleocytoplasmic transport dynamics in *Arabidopsis* root cells.

### 5.1.5 Objectives

Knockout lines *ndc1*, *gp210*, and *gp210 ndc1* were used to experiments involving LMB and PUM with the aim to characterise *NDC1* and *GP210* genes. Root elongation assays with *Arabidopsis* seedlings of *ndc1* and *gp210* (the double knockout *gp210 ndc1* had not yet been created) were performed on growth media containing LMB in an attempt to observe possible

hypersensitivity of knockout lines to the nuclear transport inhibiting drug. Growth experiments with media containing oryzalin were also conducted as a positive control of hypersensitivity with the mutant line *mor1-1*.

*Arabidopsis* cells, from knockout root seedlings, were subjected to particle bombardment to transiently express GFP-PUM or RFP-PUM. Observing PUM localisation in knockout lines in comparison to wild-type will determine possible differences in nucleocytoplasmic transport. Transformed cells were also treated with LMB to observe PUM dynamics.

## 5.2. Results

### 5.2.1 Root growth inhibition assay

Hypersensitivity of growth to inhibitor drugs were investigated with root elongation growth assays using wild-type, *mor1-1*, *ndc1*, and *gp210 Arabidopsis* lines on drug concentrations of oryzalin and LMB under 24 h light and 21°C and 30°C temperature conditions. Using the microtubule depolymerising drug oryzalin, only *mor1-1* displayed a decrease in root elongation under oryzalin at 21°C (Figure 5.1A). However, *mor1-1* exhibited a more severe inhibition effect under 30°C, showing temperature sensitivity (Figure 5.1B). As expected, the knockout lines *gp210* and *ndc1* did not display hypersensitive growth inhibition to oryzalin.

Root elongation on LMB was measured for knockout lines. LMB is a potent nuclear export inhibitor displaying cellular inhibition on *Arabidopsis* leaf tissue at 10 nM (Tillemans et al. 2006). However, root elongation on media containing 0-100 nM LMB did not display hypersensitive growth inhibition of *ndc1* and *gp210* knockout line compared with wild-type (Figure 5.2).

The potency of LMB was also tested at a high concentration (2000 nM). Since LMB is known to be unstable in certain solutions, and possibly unstable to heat (Asscher et al. 2001), it was thought that it may have been denatured when added to molten agar. To counter this, this experiment measured root elongation on filter paper. There was no significant decrease of root elongation from 0 nM to 2000 nM of LMB for all three *Arabidopsis* lines, although there was a downward trend for both *ndc1* and *gp210* relative to the wild-type control (Figure 5.3). Thus, LMB does not appear to dramatically inhibit root growth in *Arabidopsis* seedlings.

### 5.2.2 PUM transformation of plant cells

PUM proteins fused to GFP or RFP were used in particle bombardment transformations. Onion cells and *Arabidopsis* root cells were transformed by particle bombardment and transiently expressed GFP-PUM protein showed localisation in cytoplasmic and nuclear structures (Figure 5.4).

Onion cells were used for transformation to provide a positive control for the *Arabidopsis* roots. Because the onion epidermal cells are readily available and highly suitable for microscopy, they have become the standard system for gene gun transformation (Collings 2013). The nucleus of onion epidermal cells were clearly visible with transmitted light (Figure 5.5B), and excluded GFP-PUM (Figure 5.5A). Onion cells treated with 370 nM LMB for 24 h displayed accumulation of GFP-PUM protein in the nucleus, demonstrating inhibition of nuclear export of the protein with LMB (Figure 5.5B). Onion cells transformed with free YFP (a protein not actively transported through the NPC but which at 27 kDa freely diffuses) showed no nuclear accumulation after treatment with LMB (Figure 5.5D).

*Arabidopsis* cells transiently expressing GFP-PUM or RFP-PUM were also treated with LMB, and this caused nuclear accumulation of PUM in all *Arabidopsis* lines tested (wild-type, *ndc1*, *gp210*, and *gp210 ndc1*) (Figure 5.6). This indicates that although LMB had no a significant effect on root growth of *Arabidopsis* seedlings, it still inhibited nuclear export.

It was also observed while screening knockout lines transiently either expressing GFP-PUM or RFP-PUM that some cells displayed nuclear accumulation without LMB treatments (Figure 5.7C, F, G). However, no wild-type cells displayed nuclear accumulation (Figure 5.7A, B), and a proportion of cells from knockout lines also did not display significant nuclear accumulation of GFP-PUM or RFP-PUM (Figure 5.7D, E, H).

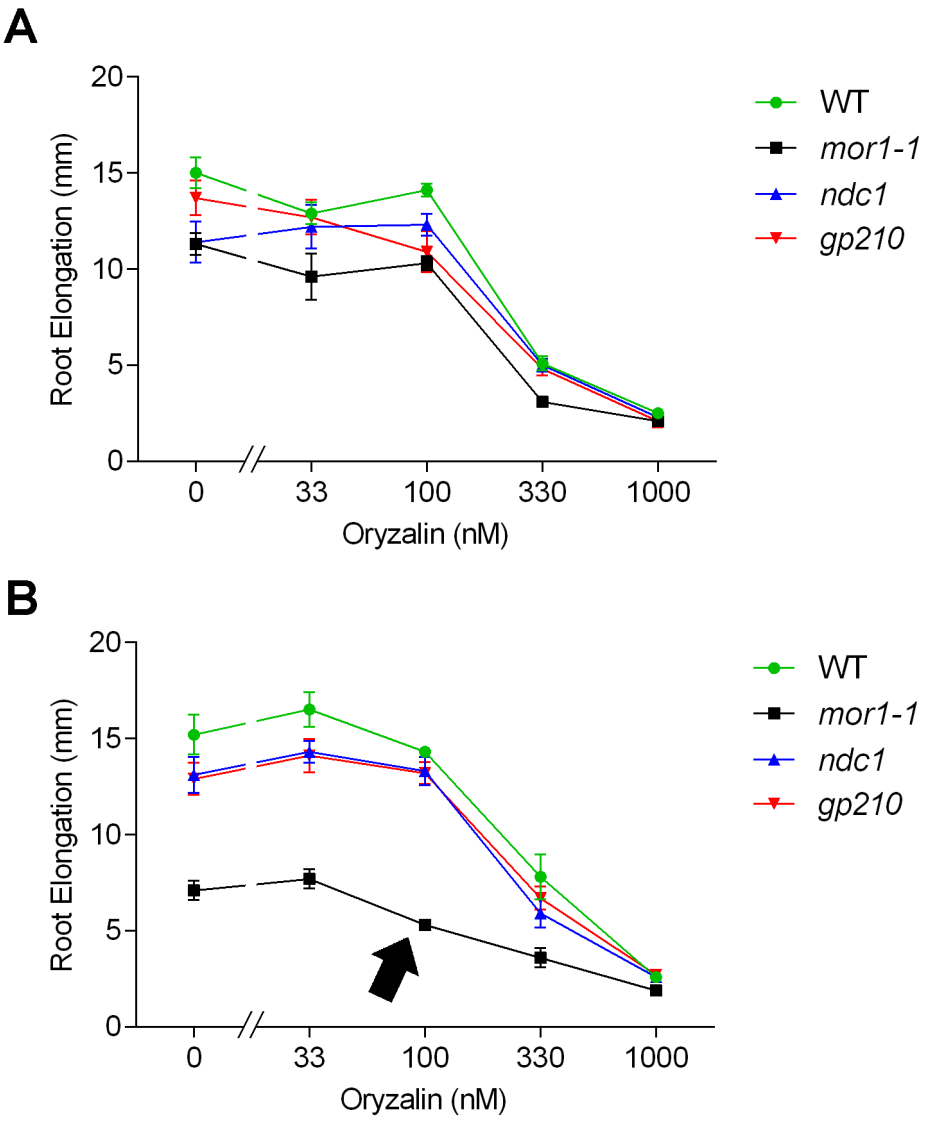


**Figure 5.1.** *Arabidopsis* root elongation of 5 d old seedlings grown for a further 48 h on 0-1000 nM oryzalin media, elongation during this time was measured.

Temperature conditions:

**A** at 21°C (permissive temperature for *mor1-1*)

**B** at 30°C (restrictive temperature for *mor1-1*)

Only *mor1-1* positive control line shows significant decrease in root growth under oryzalin concentration (arrow). Data are mean values ± SEM, n= 6.

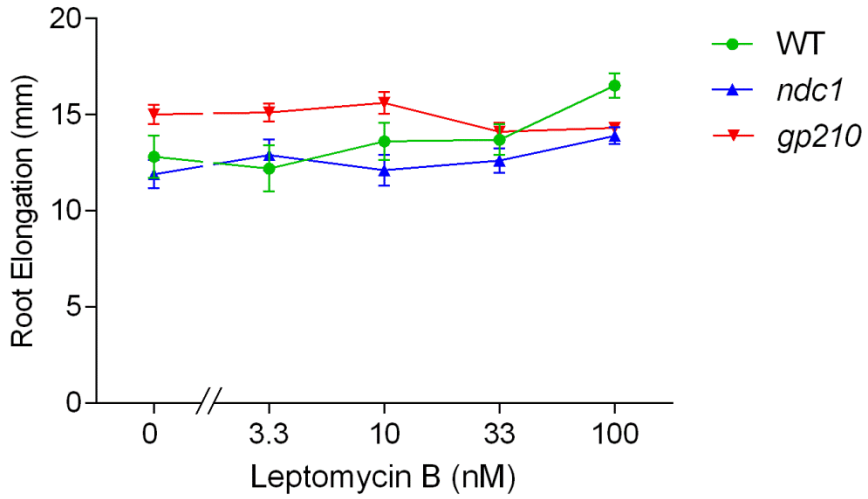


**Figure 5.2.** *Arabidopsis* root elongation of 5 d old seedlings grown for a further 48 h on 0-100 nM LMB dissolved in 1.2% agar plates at 21°C. Elongation during this time was measured, and *Arabidopsis* root growth was not susceptible to LMB. Data are mean values ± SEM, n = 10.

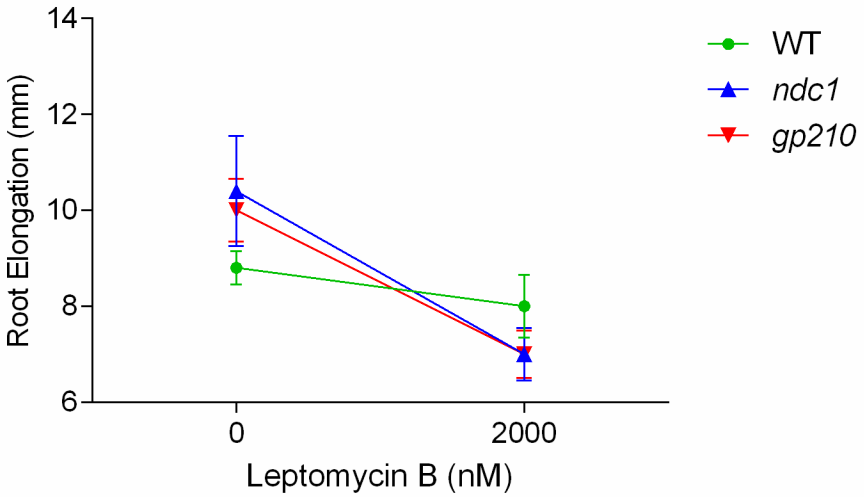


**Figure 5.3.** *Arabidopsis* root elongation of 5 d old seedlings grown for a further 72 h on +/- 2000 nM LMB containing growth paper at 21°C, elongation during this time was measured. *Arabidopsis* root growth was not susceptible significantly to LMB. Data are mean values ± SEM, n = 4.

Because the aim of these experiments was to determine whether LMB modulated nuclear transport in knockout lines, a method of quantitatively measuring fluorescence and determining the fluorescent levels in the nucleus relative to the cytoplasm was designed, with the aim in determining whether there was a significant difference in nuclear transport in *Arabidopsis* between wild-type and knockout lines. This method was developed using onion epidermal cells, and required that all images were collected using similar imaging settings so that comparison between cells might be made Confocal images of cells expressing GFP-PUM or RFP-PUM were collected, and using the quantification tools in the Leica software, regions covering the nucleus and cytoplasm were selected. The mean grey values of the marked areas were measured, and the nucleus to cytoplasm fluorescence ratio was calculated for transiently expressing onion and *Arabidopsis* cells.

Nuclear to cytoplasmic fluorescent ratios in onion cells transiently expressing GFP-PUM were calculated, and there was a significant change (P value <0.0001) in cells treated with LMB compared to untreated cells (Figure 5.8A-B). Free YFP expressing cells were used as a control and showed no change in fluorescent nucleus to cytoplasmic ratio (Figure 5.8C-D).

The fluorescent nucleus to cytoplasmic ratio of *Arabidopsis* cell lines expressing GFP-PUM or RFP-PUM were also calculated (Figure 5.9A-B). Free YFP transiently expressed in wild-type roots was used as a control, and no difference between LMB treated and untreated cells was measured (Figure 5.9C). However, there was a significant difference between the fluorescent ratios measured in LMB treated and untreated cells in wild-type and *gp210* plants (P value <0.005). No significant difference was seen between LMB treated and untreated cells for the *ndc1*plants (Figure 5.9A). The fluorescent ratio of *ndc1* cells expressing GFP-PUM (-LMB) was measured significantly higher to transiently expressed wild-type cells (-LMB) (Figure 5.9A) These data show that while *gp210* plants showed similar nuclear localisation data to wild-type plants, *ndc1* plants have a clear phenotype with more accumulation of GFP-PUM than wild-type even in the absence of LMB.

For the double knockout line *gp210 ndc1* which expressed nuclear GFP from parental crosses, RFP-PUM was used to specifically visualise PUM transportation because the measurement conditions for RFP differed from GFP. The ratios of RFP and GFP were not directly comparable, as PUM proteins are not represented in equimolar concentrations in GFP-PUM compared to RFP-PUM (Tam et al. 2010) and because of different imaging conditions. Wild-

type cells expressing RFP-PUM was used as the control for *gp210 ndc1*. A significant increase in fluorescent ratio of *gp210 ndc1* (– LMB) was measured compared to wild-type (– LMB) (P value <0.01) (Figure 5.9B). A significant increase in fluorescent ratio was also measured in LMB treated cells of *gp210 ndc1* compared to wild-type (P value <0.01) (Figure 5.9B).

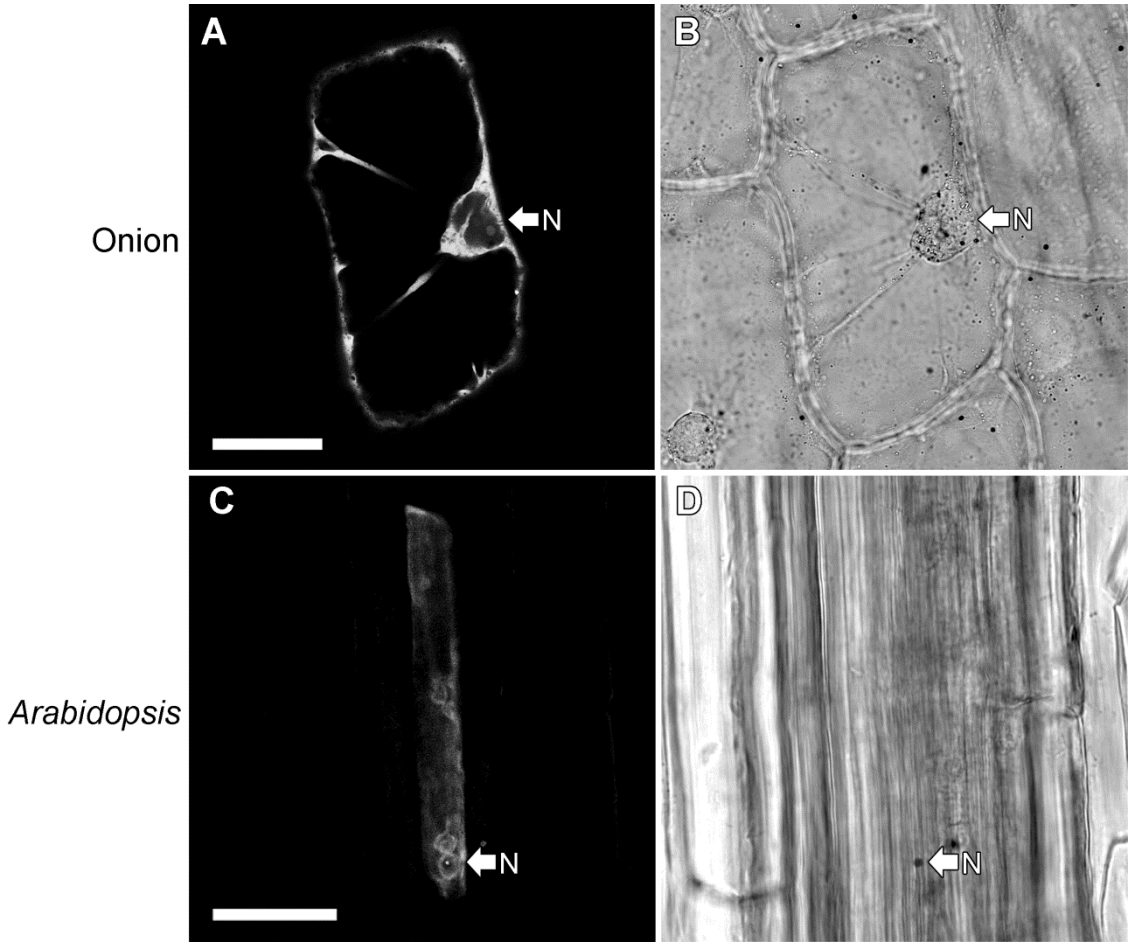


**Figure 5.4.** Onion epidermal cells and *Arabidopsis* root cells transiently expressing GFP-PUM (**A, C**) beside relative transmitted light images (**B, D**). N = nucleus.

**A** scale bar = 50 µm.

**C** scale bar = 25 µm.

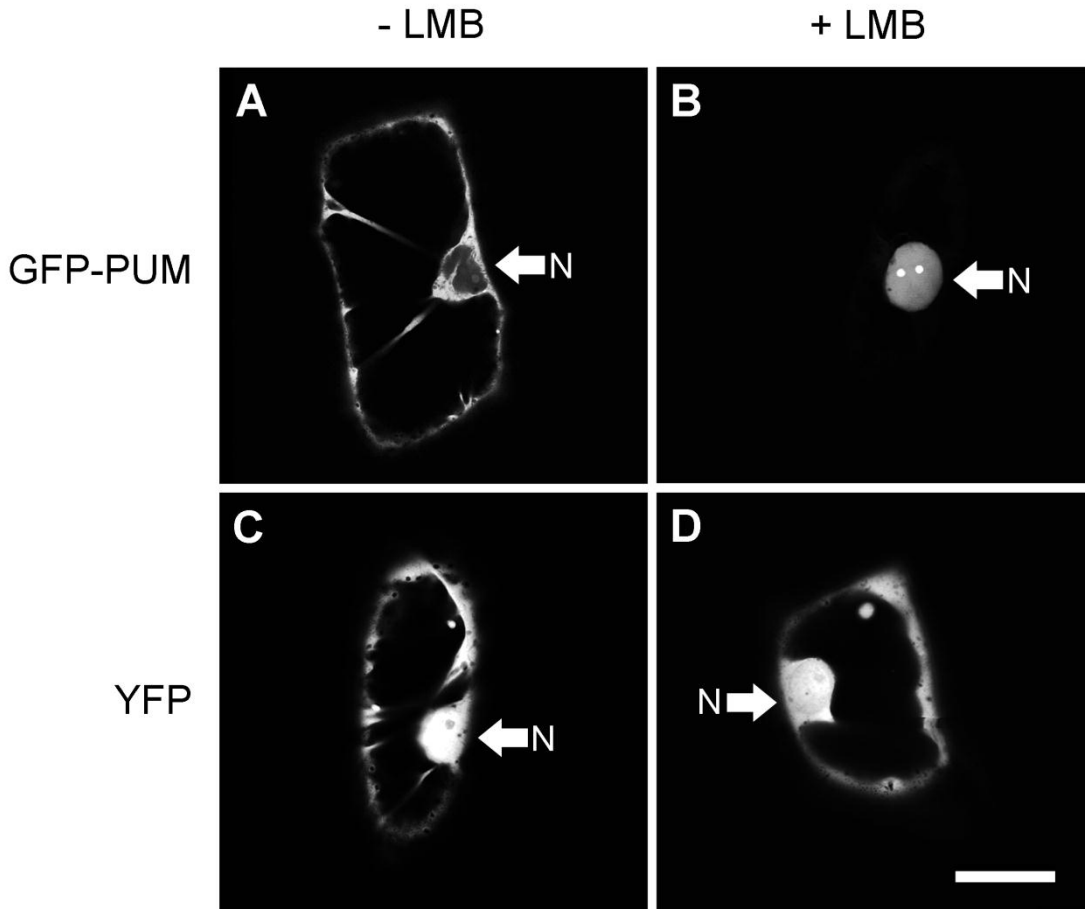


**Figure 5.5.** Onion epidermal cells transiently expressing GFP-PUM and cytoplasmic YFP, and treated with or without 370 nM leptomycin B (LMB) for 24 h. Nuclear accumulation of GFP-PUM was observed under LMB treatment but cytoplasmic YFP was not affected. N = nucleus. Scale bar = 50 µm.

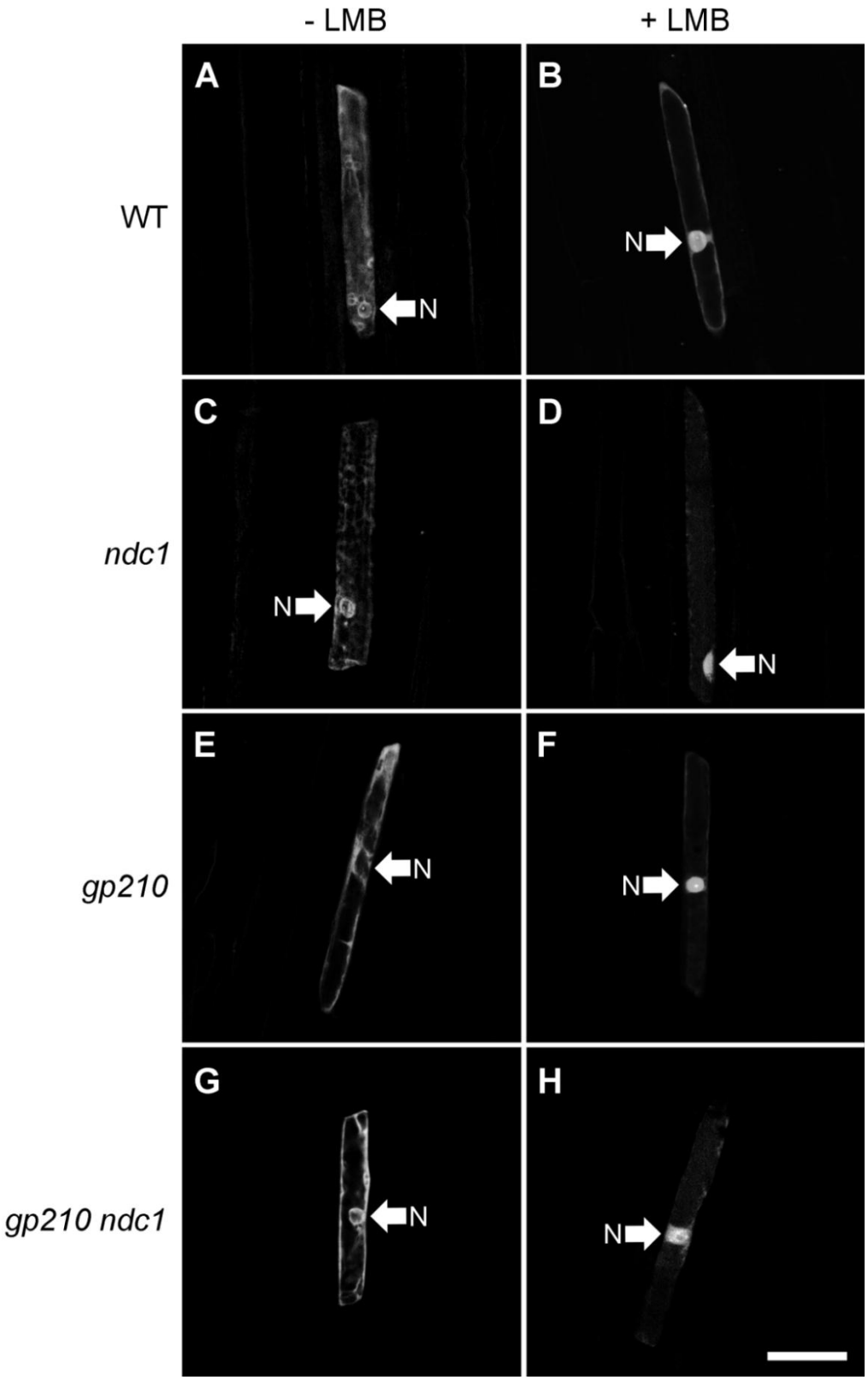


**Figure 5.6.** *Arabidopsis* 12 d old cells transiently expressing GFP-PUM (**A-F**) and RFP-PUM (**G, H**), and treated with or without 370 nM leptomycin B (LMB) for 24 h. Nuclear accumulation of GFP/RFP-PUM showed the inhibition of nuclear export by LMB. N = nucleus. Scale bar = 25 μm.

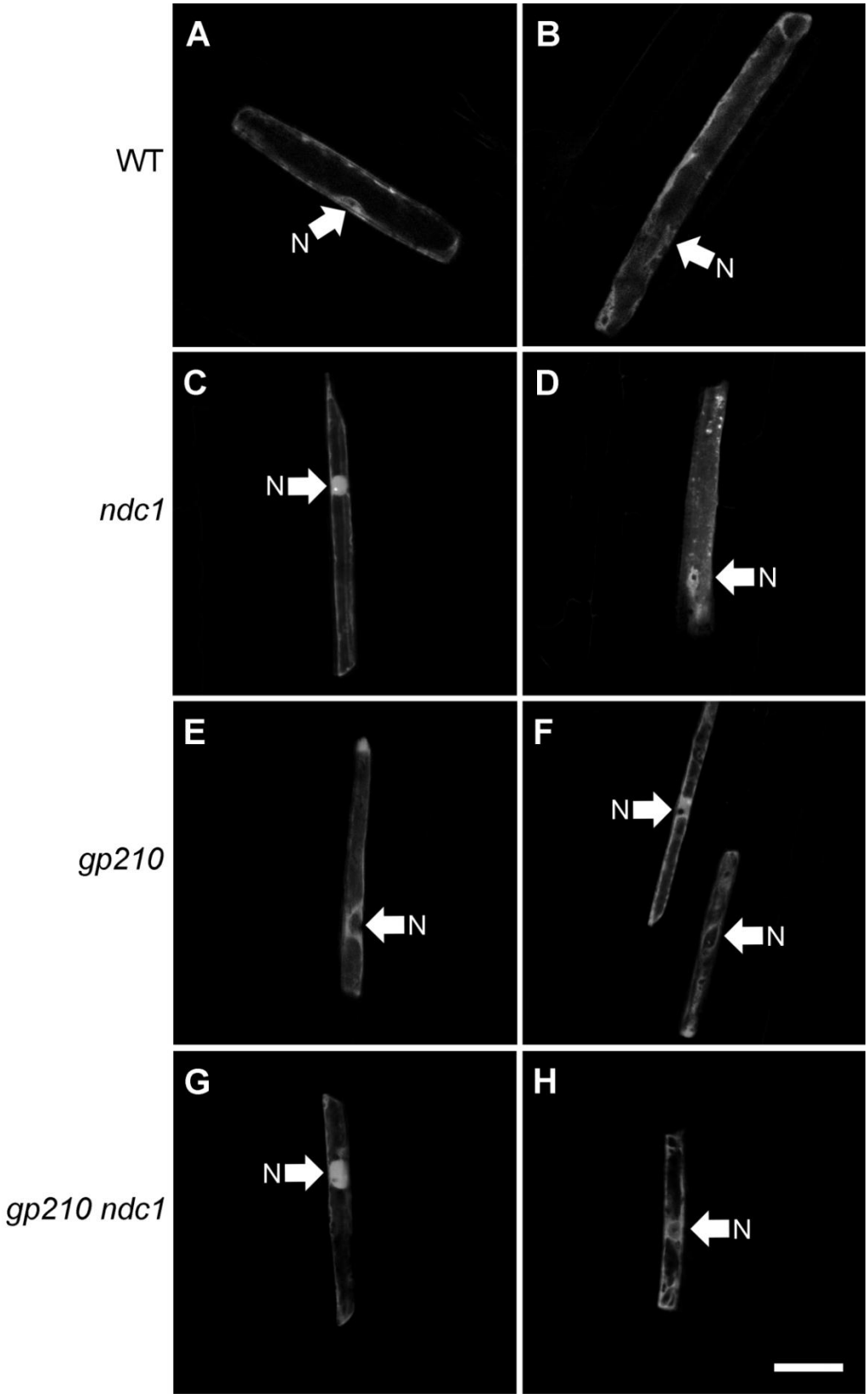


**Figure 5.7.** *Arabidopsis* 12d old roots of wild-type (WT) and knockout lines with cells transiently expressing GFP-PUM (**A-F**) and RFP-PUM (**G, H**). There were more instances of nuclear accumulation of GFP-PUM and RFP-PUM of knockout lines even in the absence of leptomycin B. N = nucleus. Scale bar = 25 µm.

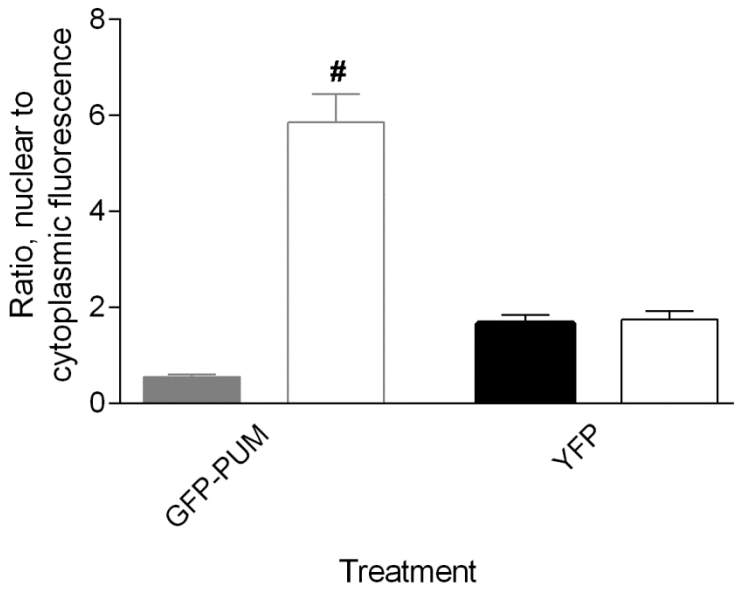


**Figure 5.8.** Mean grey value ratio of nucleus and cytoplasm fluorescence in onion cells transiently expressing GFP-PUM and free YFP. Filled bars are water control while open bars are 370 nM LMB (24 h). The increased value of GFP-PUM treated with LMB indicates fluorescent accumulation in the nucleus. The YFP control displayed no effect. Data are mean values ± SEM, n = 10. # indicates a significant difference caused by addition of LMB, P value <0.0001.

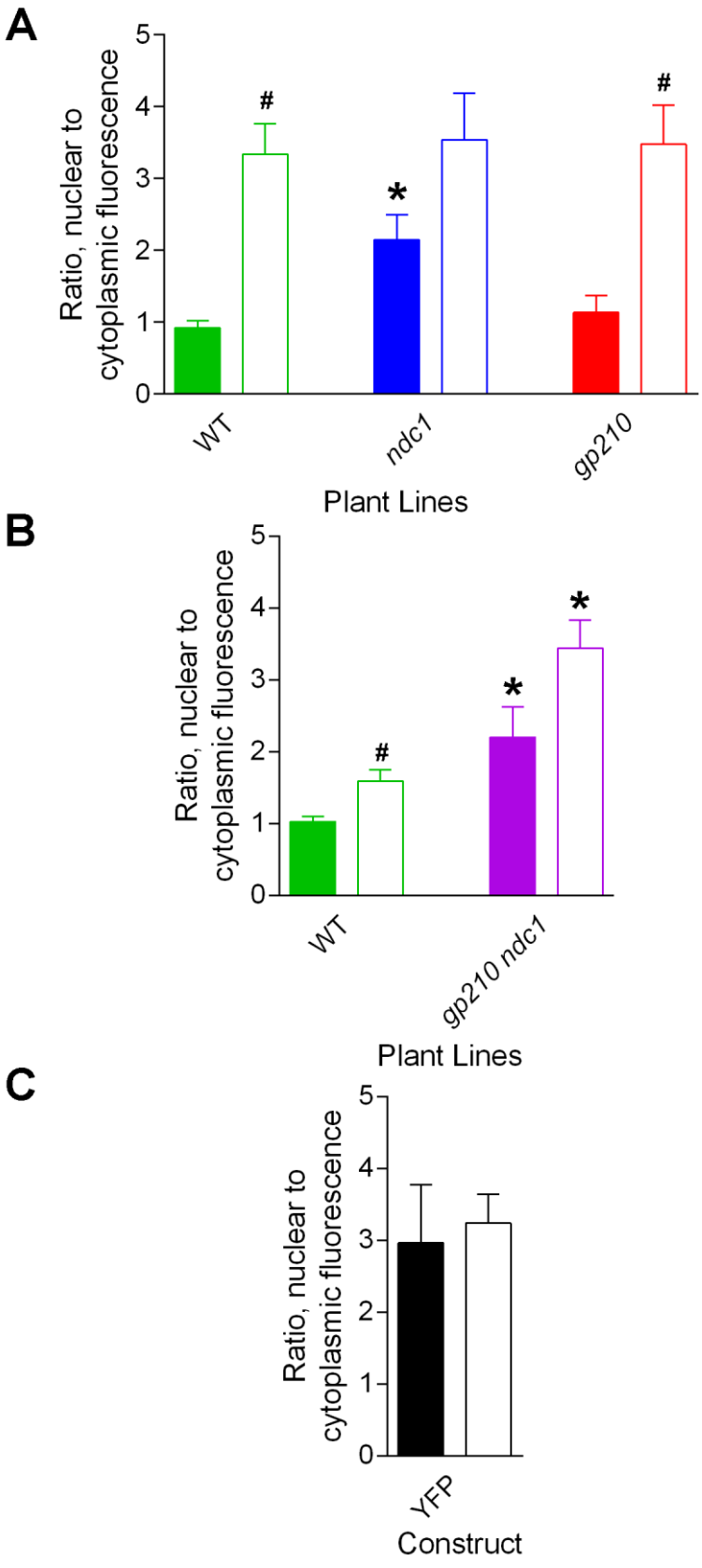


**Figure 5.9. Continued next page.**

**Figure 5.9.** Mean grey value ratio of nucleus and cytoplasm fluorescence in different *Arabidopsis* cell lines expressing:

**A** GFP-PUM.

**B** RFP-PUM.

**C** YFP control in wild-type.

Filled bars are water control while open bars are 370 nM LMB (24 h). All lines expressing GFP-PUM and RFP-PUM displayed significant change in ratio after LMB treatment except for the *ndc1* and *gp210 ndc1* knockouts and free YFP control. Data are mean values ± SEM, n = 10.* significant difference to wild-type plant line, P value <0.01.

# indicates a significant difference caused by addition of LMB, P value <0.005.

## 5.3. Conclusions

The nuclear export inhibitor LMB was shown to not significantly affect root growth in *Arabidopsis* seedlings. *Arabidopsis* seedlings growing on 2000 nM displayed no significant decrease in growth but both knockout lines exhibited a sharper decrease in growth than wild-type. These results show that while LMB may not significantly affect the root growth of *Arabidopsis* seedlings, there may be a slight sensitivity to the drug in the knockout lines *ndc1* and *gp210.*

Nuclear export in *Arabidopsis* showed significant responses to LMB, with nuclear accumulation of nuclear transported protein PUM fused to GFP or RFP, present in all *Arabidopsis* lines. More interestingly, the knockout line *ndc1* and double knockout *gp210 ndc1* had significant differences in nucleocytoplasmic transport even in the absence of LMB. Significantly higher fluorescent nucleus to cytoplasmic ratios was observed in *ndc1* and *gp210 ndc1* cells compared to wild-type cells. There were also no significant differences observed between untreated and LMB-treated fluorescent ratios for *ndc1* and *gp210 ndc1*. These results indicate a lack of nuclear export in *ndc1* and *gp210 ndc1* and more mild response to LMB.

# Chapter 6

# Discussion

In animal and fungal systems, multiple proteins are required to anchor the nuclear pore into the nuclear envelope. Consistent with the nucleus being the fundamental defining feature of eukaryotes, and with the nuclear pore being indispensable for nuclear functioning, bioinformatic analyses have demonstrated that many of the pore proteins, including the transmembrane ring anchoring mechanism, were present in the last common ancestor of eukaryotes (Neumann et al. 2010).

Homologues of two such anchoring proteins, *GP210* and *NDC1,* were previously identified in *Arabidopsis* and have been investigated through reverse genetics, loss-of-function approach in this study. In this general discussion, I investigate the implications for understanding the plant nuclear pore and its anchoring mechanism of the relatively minor effects on plant growth and developments observed in *Arabidopsis* plants in which one or both of *NDC1* and *GP210* are knocked out, and conclude that other subunits of the anchoring system have yet to be identified and that, as in animal and fungal systems, multiple proteins are required for anchoring the nuclear pore to the nuclear envelope.

## 6.1 Knockout line characterisation

### 6.1.1 *gp210*

The transmembrane anchoring-Nup GP210 is present in the vertebrate NPC, although it is not found in yeast where other fungal-specific anchoring proteins such as Pom34 and Pom152 are present (reviewed in Strambio-De-Castillia et al. 2010). While GP210 was found to be present in the last common eukaryotic ancestor, it appears to have been subsequently lost in fungi (Mans et al. 2004; Neumann et al. 2010). The importance of GP210 in vertebrates does not

appear to be in nucleocytoplasmic transport but rather in cell differentiation. In mouse myoblasts and embryonic stems cells, loss of GP210 through RNAi does not affect nuclear transport. It is, however, required for the induction of genes essential in differentiation, with cell death observed under differentiation conditions (D'Angelo et al. 2012). In *C. elegans* GP210 is important for efficient NPC disassembly and nuclear envelope breakdown as demonstrated by RNAi and mutation of *C. elegans* embryonic cells (Galy et al. 2008).

*GP210* was identified in *Arabidopsis* as the gene At5g40480 through bioinformatic analysis (Neumann et al. 2010) and the GP210 protein was subsequently confirmed to be part of the *Arabidopsis* NPC through co-immunoprecipitation with *Arabidopsis* Nup RAE (RNA export factor 1) fused to GFP (Tamura et al. 2010). The subcellular localisation of *GP210* in *Arabidopsis* was confirmed at the nuclear envelope through stable expression of GP210-GFP (Tamura et al. 2010). However, no loss-of-function studies for *GP210* have been published in the literature. Preliminary analyses, however, have been published online. On the TAIR database, the At5g40480 gene is annotated as embryo defective, with "a note that developmental arrest of mutant embryos occurs at preglobular stage" (http://arabidopsis.org/servlets/TairObject?id=132136&type=locus, accessed June 19th 2013). This gene is also listed in a database of embryo defective genes (http://www.seedgenes.org/SeedGeneProfile?geneSymbol=EMB+3012, accessed June 19th 2013) in which the protein is listed as functioning in nuclear protein export. Perhaps significantly, the data presented on this site refer to gene knockouts conducted in the Ws (Wassilewskija) background rather than the Columbia background used as in this study. These unpublished observations stand in stark contrast to the limited effects of *GP210* knockouts seen in this study.

In this study, homozygous *gp210* knockout lines were isolated, but the embryo defective phenotype was not observed. Not only were no embryo abortions detected (Figure 4.16), but in there was no deviation from the classic 1:2:1 Mendelian segregation ratios when heterozygous insertional lines were self-pollinated (Tables 4.2 – 4.4). It is possible that the annotation on the TAIR and SeedGenes websites may be in error or that there are differences caused by different genetic backgrounds.

In summary, no significant differences were seen in the plant development of *gp210* when compared to wild-type lines, and no differences were observed in nucleocytoplasmic transport

(Figure 5.9A) This indicates that *GP210* does not appear to be essential in the development of the plant.

Plant fasciations were evident in at least one *gp210* plant, and potentially in another plant with a T-DNA insert in *GP210* at an unknown location, although no connection between the knockout of the gene and the phenotype could be established. Instead, uncontrolled stresses on the fasciated plants were deemed to be the cause since the fasciation phenotype can be generated by stresses such as insect attack, mechanical pressure and/or tension, time and density sowing, temperature fluctuation, mineral deficiency and biotic stresses caused by microbial infections (Gorter 1965; Iliev and Kitin 2011). Although the phenotype could not be replicated, it is possible that the *gp210* knockout caused the plant to be more susceptible to fasciation under these specific (but uncertain) stresses. This might be investigated further.

### 6.1.2 *ndc1*

While the anchoring-Nup NDC1 has not yet been characterised in plants, its roles in anchoring the nuclear pore in vertebrates and yeast has been described in detail (reviewed in Strambio-De-Castillia et al. 2010). NDC1 plays important roles in the viability of organisms. The loss-of-function of NDC1 in *C. elegans* causes severe defects and high larval and embryonic mortality (Stavru et al. 2006a). In yeast, NDC1 is not only a component of the NPC but also the spindle pole body (the yeast equivalent of the centrosome) which is embedded in the nuclear envelope. Loss of NDC1 function in yeast mutant *ndc1-1* shows lethality (Hetzer et al. 2005; Winey et al. 1993). And in humans, NDC1 is the main anchor of ALADIN and the loss of this interaction is involved in the pathogenesis of disease triple-A syndrome, an autosomal recessive congenital disorder (Kind et al. 2009).

Bioinformatic analysis identified that NDC1, initially thought only to be present in opisthokonts (Bapteste et al. 2005), is also present in plants with the gene At1g73240 identified as its *Arabidopsis* homologue (Neumann et al. 2010).

The growth and development of the *Arabidopsis ndc1* knockout showed mild but significant differences to wild-type plants. There were significant changes in the number of leaves at the bolting and flowering stage of the plants under both sets of lighting conditions investigated (Figure 4.2 & 4.4). There were also significant deviations from the standard Mendelian 1:2:1 distributions for selfed heterozygous *NDC1* knockouts (Tables 4.2 – 4.4) that suggest defects

in *ndc1* pollination or embryonic development. These subtle changes in growth and development may derive from the subtle changes in nucleocytoplasmic transport (Figure 5.9A). The inhibition in nuclear export of proteins observed in *ndc1* may restrict the movement of regulating proteins involved growth and development out of the nucleus. Further characterisations of these effects should be conducted.

While the fasciation phenotype was observed in an *ndc1* line, no connection between the knockout the phenotype was found. As this phenotype was observed in *ndc1* and *gp210* knockout lines it is a possibility that under particular stresses anchoring-Nups may be more susceptible to fasciation, as discussed in the preceding section.

### 6.1.3 *gp210 ndc1*

Loss-of-function experiments of GP210 and NDC1 simultaneously have been carried out before as combinatorial RNAi experiments in HeLa K cells (Mansfeld et al. 2006). An interaction had been observed between GP210 and NDC1 through RNAi-mediated depletion of both Nups causing a severe decrease in number of FG-repeats (mediators of active nucleocytoplasmic transport) in cells. This was an synergistic interaction as RNAi-mediated depletions of GP210 and NDC1 individually were not as severe, suggesting a partial overlap of NPC function and dynamic (Mansfeld et al. 2006). In plants, double T-DNA knockouts of closely related Nups have yield combinatorial results with *sar1 sar3* generating enhanced phenotypic defects greater than the single mutants (Parry et al. 2006). The generation of the *gp210* and *ndc1* double knockout should detect whether there is any overlap in gene function, and demonstrate how robust the plant NPC is to the simultaneous loss of these two anchoring-Nups.

Through the use of crossing techniques with nuclear targeted GFP lines, *gp210 ndc1* double knockouts were generated for analysis. The seed viability of *gp210 ndc1* double knockouts was investigated and observed to be normal with no difference detected of seed abortions compared with wild-type. The knockout of both anchoring-Nups does not show a lethal affect in *Arabidopsis.* The nuclear morphology of *gp210 ndc1* in nuclear GFP expressing cells was similar to control cells (Figure 4.18). This indicated the concurrent knockout of these two genes does not affect the structure and integrity of the cell nuclei. This may be accounted that

NPC biogenesis is an extremely fault tolerant process as shown in other NPC complexes such as in *C. elegans* (Stavru et al. 2006a).

Nucleocytoplasmic transport in *gp210 ndc1* had significant differences compared to wild-type, indicating a lack of nuclear export (Figure 5.9B). This significant difference is also seen for *ndc1* but not for *gp210* (Figure 5.9A). The inhibition of nuclear export seen in *gp210 ndc1* double knockout can be accounted for in *ndc1* as *gp210* displayed no difference in nucleocytoplasmic transport and there was no enhanced observation of nuclear export seen in these cells (Figure 5.9).

## 6.2 Future research directions

There are currently significant gaps in understanding of the plant NPC, the investigation of which has been limited when compared to some other eukaryotes. To extend the current study into the anchoring of the plant NPC, and the role that *NDC1* and *GP210* play in this process, several different lines of investigation would need to be taken.

(i) Bioinformatic studies have indicated that At1g73240 and At5g40480 are the *Arabidopsis* homologs to *NDC1* and *GP210* (Neumann et al. 2010). GFP fusions to At5g40480 have confirmed that this protein localises to the nuclear membrane of *Arabidopsis* consistent with its identification as GP210 (Tamura et al. 2010). Similar studies, however, yet to be conducted for NDC1. Although it was anticipated that the phenotypic analysis of plants in this study would help confirm the identification of At1g73240 as *NDC1*, possible through observations of the synergistic effects between *ndc1* and *gp210* knockout lines, the results have not actually confirmed this. It is, therefore, critical that the localisation of the At1g73240 gene product would be needed to confirm its characterisation. The localisation to the nuclear envelope of transiently or stably expressed fusion protein between GFP and At1g73240 gene product would confirm the identification of At1g73240 as *NDC1*. It was initially intended that such GFP fusions were to be made part of this project. However, no full length cDNA for *NDC1*were available from the stock centres (although a partial cDNA for the gene was available from the RIKEN centre in Japan), it was decided that the phenotyping approach would be more profitable.

(ii) Finding other Nups that facilitate transmembrane anchoring, along with *ndc1* and *gp210*, is also critical for understanding the anchoring system. The viability and mild phenotypic responses seen in *ndc1, gp210,* and *gp210 ndc1* implies that other Nups in the anchoring system have not been identified. The NPC anchoring system in other eukaryotic kingdoms comprises proteins not yet found in plants. These are Pom121 in vertebrates, and Pom152 and Pom34 in yeast (Strambio-De-Castillia et al. 2010). By analogy, other anchoring protein(s) should be present in plants although whether these would be unrecognised homologues of Pom121, Pom152, or Pom34, or a novel protein unique to plants, cannot yet be known. Finding unidentified anchoring-Nups in plants should expose the redundant functions seen in *ndc1 gp210*.

Bioinformatic screening techniques were used in identifying NDC1 and GP210 through sequence similarity with vertebrate Nups (Neumann et al. 2010). This method may have limited the identification by excluding anchoring-Nups that exhibit a significant difference in sequences. Immunoprecipitation technique using a known *Arabidopsis* Nup (RAE, RNA export factor 1) fused to GFP identified 200 proteins under that co-immunoprecipitated with RAE using antibodies raised against GFP. The 200 proteins were compared to metazoan sequence databases, and 22 *Arabidopsis* Nups were identified by sequence similarity (Tamura et al. 2010). This experiment demonstrates that the plant NPC is compared of various proteins that have yet to be fully described (Wiermer et al. 2012).

A similar immunoprecipitation technique could be employed with either NDC1-GFP or GP210-GFP to find possible anchoring-Nups that were not immunoprecipitated with RAE, such experiments would use GFP-NDC1 or GFP-GP210 expression in appropriate knockout backgrounds, to detect proteins immunoprecipitated with the GFP-fusion protein using antibodies to GFP. Alhough this method still relies on sequence similarity to identify Nups. However, another way to screen these precipitated proteins is through co-expression analysis, where genes of similar functions often behave similarly with respect to their associated transcript, protein or metabolite profiles and are measured by –omics technologies (Bolle et al. 2011). This method combines gene expression measurements from a large number of experiments to improve statistics (Bolle et al. 2011). Co-expression analysis can be used to predict the function of genes based on the similarity of their expression patterns (Loraine 2009;

Usadel et al. 2009). This was shown in the nuclear genes for chloroplast proteins involved in photosynthesis or plastid gene expression by exhibiting high levels of co-expression at the transcript level (Biehl et al. 2005). This finding was exploited to systematically characterise by reverse genetics genes of unknown function that exhibited photosynthesis gene-like transcriptional profiles leading to the identification of PGRL1, a central component of cyclic electron flow around photosystem I (DalCorso et al. 2008). Co-expression profile analysis of unknown genes to NDC1 and GP210 is a potential method in the discovery of new anchoring-Nup genes. However, as NDC1 and GP210 are expressed in all cell types, being fundamental for nuclear function, such co-expression analysis might be more difficult.

A further technique for identifying novel transmembrane anchoring-Nups in plants would be to detect protein-protein interactions with existing Nups by yeast-two hybrid screens. This technique can be performed with two fusion proteins prepared for the known and proposed Nups (from either co-immunoprecipitation or co-expression analysis) fused to transcription factor gene (e.g. Gal4) and reporter gene (e.g. LacZ), when the proteins interact transcription is initiated and the reporter gene activates (Young 1998). The NPC-associated proteins in *S. cerevisiae* Rip1p and Crm1p were identified in nuclear export using yeast-two hybrid screens because they interact with the export protein Rev NES. These proteins were also shown to contribute to Rev-mediated export (Neville et al. 1997; Stutz et al. 1995).

(iii) Further experiments are also required to understand the observed *ndc1* knockout phenotypes, notably the reduced nuclear export and non-Mendelian segregation. Experiments in detailing ovule and pollen development would be step in determining the reason for change in genotype distribution frequency of heterozygous and homozygous *ndc1*. Such experiments were performed in the double knockout line *AtREV3* and *AtPOLH*, mutants of translation polymerase genes, which yielded non-Mendelian F2 genotype distributions. It was found that T-DNA linked translocations were involved (Curtis et al. 2009).

(iv) There are also alternative methods of observing the knockout or knockdown of Nup orthologs in *Arabidopsis*. With techniques using RNAi and miRNA in generating loss-of-function available (Schwab et al. 2006). RNAi has contributed to characterising

GP210 in vertebrates with RNAi-mediated depletion of GP210 in both human cultured cells and *C. elegans* embryos affecting cell viability (Cohen et al. 2003). RNAi was also used on NDC1 causing severe defects and very high larval and embryonic mortality in *C. elegans*. RNAi-dependent NDC1 depletion in human HeLa cells was found to interfere in the assembly of FG-repeat Nups into the NPC and also result in the mislocalisation of Nup ALADIN, involved in triple-A syndrome disease (Stavru et al. 2006a; Yamazumi et al. 2009). With RNAi techniques now readily available for *Arabidopsis*, there are opportunities in characterising the loss-of-function of anchoring-Nups apart than T-DNA insertion.

There has been a challenge that the vast majority of gene knockouts in *Arabidopsis* do not give rise to obvious phenotypes, functional characterisation by –omics-type analysis or simultaneous inactivation/down regulation of more than one gene (Bolle et al. 2011). Nups are known to be able to facilitate the function of close relatives that have been knocked out (Parry et al. 2006). The plant NPC is known to be composed of various subunits that have yet to be described (Wiermer et al. 2012), and with NPC anchoring in other eukaryotic kingdoms composed with anchoring subunits not yet found in plants finding unidentified anchoring-Nups in plants should expose the redundant functions seen in *ndc1 gp210*.

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# Appendix

## A.1 BLAST alignments

### A.1.1 *NDC1* wild-type PCR product BLAST alignment to At1g73240

*Arabidopsis thaliana* chromosome 1, complete sequence
Sequence ID: ref|NC_003070.9|Length: 30427671Number of Matches: 1
Alignment statistics for match

| Score | Expect | Identities | Gaps | Strand |
|---|---|---|---|---|
| 1892 bits(1024) | 0.0 | 1024/1024(100%) | 0/1024(0%) | Plus/Plus |

Range 1: 27543992 to 27545015
Features: hypothetical protein

```
Query  11        ATAGCATTATCGTCGACAACACATAAAGCACACCAGATAGCTTCAAAGCTTGTGTTAGAG  70
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Sbjct  27543992  ATAGCATTATCGTCGACAACACATAAAGCACACCAGATAGCTTCAAAGCTTGTGTTAGAG  27544051

Query  71        CCGAAGGAAGACCAATCTTGAAGCTGAAGAACGGTGGACGCTGTTTTGAAAAAACACAAG  130
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Sbjct  27544052  CCGAAGGAAGACCAATCTTGAAGCTGAAGAACGGTGGACGCTGTTTTGAAAAAACACAAG  27544111

Query  131       TAAGAATCAGCAAAGATACAAAATTGATGAAACATTCCATAAATCAAGAAGCAAAGACAC  190
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  27544112  TAAGAATCAGCAAAGATACAAAATTGATGAAACATTCCATAAATCAAGAAGCAAAGACAC  27544171

Query  191       AAAACCTGAATGATAGGAAACTCCAAAACCCATCGTTGCTTAGTAACAAAGAGGACTGCG  250
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  27544172  AAAACCTGAATGATAGGAAACTCCAAAACCCATCGTTGCTTAGTAACAAAGAGGACTGCG  27544231

Query  251       TAAAGCAATCCAGTCACTAATCCCCTAAACCCTATTCTTCCAATGGGTCCAAACGAATCA  310
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Sbjct  27544232  TAAAGCAATCCAGTCACTAATCCCCTAAACCCTATTCTTCCAATGGGTCCAAACGAATCA  27544291

Query  311       CTAGACAAGCAAAACACAGCAGAGGAGCAGAATCCAGCGAGGGAAGTAGCGCAGACGAAG  370
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Sbjct  27544292  CTAGACAAGCAAAACACAGCAGAGGAGCAGAATCCAGCGAGGGAAGTAGCGCAGACGAAG  27544351

Query  371       AGCACGATGCGTGAAGATACGCGAGCACGGCGGCGGAACTCGGGATCCGAGAGGTGACGG  430
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Sbjct  27544352  AGCACGATGCGTGAAGATACGCGAGCACGGCGGCGGAACTCGGGATCCGAGAGGTGACGG  27544411

Query  431       TGAAGACCGACAGCGAAttggagaggggagagaggagaatctggagtcggagaagagagg  490
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Sbjct  27544412  TGAAGACCGACAGCGAATTGGAGAGGGGAGAGAGGAGAATCTGGAGTCGGAGAAGAGAGG  27544471

Query  491       agagctagtgagacggagaatagaagctgtgaagaatggaagacgaggaaagtgaagaga  550
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Sbjct  27544472  AGAGCTAGTGAGACGGAGAATAGAAGCTGTGAAGAATGGAAGACGAGGAAAGTGAAGAGA  27544531
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Query  551       aaggaaatgattgaagtggttgagaagaggaaaatgttgaagaagaagaagatggctgat  610
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Sbjct  27544532  AAGGAAATGATTGAAGTGGTTGAGAAGAGGAAAATGTTGAAGAAGAAGAAGATGGCTGAT  27544591

Query  611       gaaggaatcgattgccagataagaaaagctgcgaagcggtggctCACAACCGTCTCCGGC  670
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  27544592  GAAGGAATCGATTGCCAGATAAGAAAAGCTGCGAAGCGGTGGCTCACAACCGTCTCCGGC  27544651

Query  671       ACCGGAGGAGAAGGCATAATCGCCGGCGATTGCTTTTGCCCCTTTTGTGATTAAGGTTTT  730
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  27544652  ACCGGAGGAGAAGGCATAATCGCCGGCGATTGCTTTTGCCCCTTTTGTGATTAAGGTTTT  27544711

Query  731       ATTAAATTAATTTGAAATGAGTTTCGTGTCAGAGCTTGAGCGGGGCCGTGAAGTCAAGTG  790
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Sbjct  27544712  ATTAAATTAATTTGAAATGAGTTTCGTGTCAGAGCTTGAGCGGGGCCGTGAAGTCAAGTG  27544771

Query  791       ACTGAGCCAACGAAGACGAAGAAGAAGGTAAGCGAAAGAGAGACAGGTTAGGGCTAGTGA  850
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Sbjct  27544772  ACTGAGCCAACGAAGACGAAGAAGAAGGTAAGCGAAAGAGAGACAGGTTAGGGCTAGTGA  27544831

Query  851       GCCGCGTTTTGTTCACGCTATCTTTAACAAGGCTTGTGTAAGCCCATTGGGCCGAGAATT  910
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Sbjct  27544832  GCCGCGTTTTGTTCACGCTATCTTTAACAAGGCTTGTGTAAGCCCATTGGGCCGAGAATT  27544891

Query  911       CATTATTTGTCATTTTAGCGTTGCAACTTGCAAGGATAGCCAAATTAGTGAGATATCAAA  970
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Sbjct  27544892  CATTATTTGTCATTTTAGCGTTGCAACTTGCAAGGATAGCCAAATTAGTGAGATATCAAA  27544951

Query  971       TGTTTTTCTTGAAACTTCTTACAACTGTTTTGATAATTTGTATAGAAGAGAGCTAATAAT  1030
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  27544952  TGTTTTTCTTGAAACTTCTTACAACTGTTTTGATAATTTGTATAGAAGAGAGCTAATAAT  27545011

Query  1031      AGAC  1034
                  ||||
Sbjct  27545012  AGAC  27545015
```

### A.1.2 *GP210* wild-type PCR product BLAST alignment to At5g40480

*Arabidopsis thaliana* chromosome 5, complete sequence
Sequence ID: ref|NC_003076.8|Length: 26975502Number of Matches: 1
Range 1: 16215369 to 16216547
Alignment statistics for match

| Score | Expect | Identities | Gaps | Strand |
|---|---|---|---|---|
| 2052 bits(1111) | 0.0 | 1165/1189(98%) | 12/1189(1%) | Plus/Plus |

Features: protein embryo defective 3012

```
Query  17        ACTGCTGTTC-TTTGTTTG-ATTTAAGTTTCTTCCTTTACCGAGTTATGTCCAGAGGATT  74
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Sbjct  16215369  ACTGCTGTTCATTTGTTTGCATTTAAGTTTCTTCCTTTACCGAGTTATGTCCAGAGGATT  16215428

Query  75        TATTGTAGATGATATTGCATGTATGTTCAACATAATAATTTCCTTTGTGTTGATTGCAGA  134
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Sbjct  16215429  TATTGTAGATGATATTGCATGTATGTTCAACATAATAATTTCCTTTGTGTTGATTGCAGA  16215488

Query  135       CGGATGACATAAAACTGTATGGAAAAGATTCAGATTATTGGAAAATCGTCTCACTGCCAG  194
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  16215489  CGGATGACATAAAACTGTATGGAAAAGATTCAGATTATTGGAAAATCGTCTCACTGCCAG  16215548

Query  195       ATGAACTTTCCTCTGAATATGGTCAGCGAAATTCTAGAATTTTGAACGCAATCTCACCAG  254
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Sbjct  16215549  ATGAACTTTCCTCTGAATATGGTCAGCGAAATTCTAGAATTTTGAACGCAATCTCACCAG  16215608

Query  255       GATTAGGAGAGCTGACATCTACATTGACTTACTTCAGTGGGCATCAAGAGTCAAAAGAGG  314
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Sbjct  16215609  GATTAGGAGAGCTGACATCTACATTGACTTACTTCAGTGGGCATCAAGAGTCAAAAGAGG  16215668

Query  315       TGAGAAAATTATATATCTTTGGAAAACCTTTTGTCTCTTTCCATTTGTTGGTACTGCAAG  374
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Sbjct  16215669  TGAGAAAATTATATATCTTTGGAAAACCTTTTGTCTCTTTCCATTTGTTGGTACTGCAAG  16215728

Query  375       AGTAACAACTTTGTCTCTAGCAGGTTCTCAAGGTTGTCCAAGAAATTAGGGTTTGTGAAA  434
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Sbjct  16215729  AGTAACAACTTTGTCTCTAGCAGGTTCTCAAGGTTGTCCAAGAAATTAGGGTTTGTGAAA  16215788

Query  435       AAGTGCAGTTCACATTGAACAGTGAAGATGACACACCTAAGGTTCTACTCCCATGGACCC  494
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Sbjct  16215789  AAGTGCAGTTCACATTGAACAGTGAAGATGACACACCTAAGGTTCTACTCCCATGGACCC  16215848

Query  495       CTGCTGTTTATCAGGAGATGGAGCTAATTGTGACAGGAGGTTAGTTCCCAATCTATGGTT  554
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Query  555       CTCGTGACCTATTCTTTTTTAGTTTGAAAACCATGGTGACTTGATGATTGTTCCTCACTG  614
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Sbjct  16215909  CTCGTGACCTATTCTTTTTTAGTTTGAAAACCATGGTGACTTGATGATTGTTCCTCACTG  16215968

Query  615       TAGGTTGTGCAAAAGCATCGAGTGACTACAAGTGGTTTACTTCAGATATAAGCATTTTGT  674
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Sbjct  16215969  TAGGTTGTGCAAAAGCATCGAGTGACTACAAGTGGTTTACTTCAGATATAAGCATTTTGT  16216028

Query  675       CGGTGTCAGCTTATGGAATTATCCAGGCAAAGAGGCCCGGTATAGCCACTGTGAAGGTGG  734
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Sbjct  16216029  CGGTGTCAGCTTATGGAATTATCCAGGCAAAGAGGCCCGGTATAGCCACTGTGAAGGTGG  16216088

Query  735       TGTCGACTTTCGATTCACAAAATTTTGATGAGGTACATTGGCTTTATTGCATATTATCCA  794
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Query  795       TCAGTTGATGCTTCGACAAATAGAGAAAATATATATTTTAAATGCTCAAACTCGTTTGTG  854
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Query  855       ACTTATCATGCTATGGCCCTACTGAAAATCGTGGGGCTTCAACTGATTATTGTGTTTTCC  914
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Sbjct  16216209  ACTTATCATGCTATGGCCCTACTGAAAATCGTGGGGCTTCAACTGATTATTGTGTTTTCC  16216268

Query  915       ATAGGTTATTGTTGAAGTTTCCATTCCATCCTCTATGGTTATGTTGCAAAACTTCCCAGT  974
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Query  975       AGAGACAGTTGTTGGATCACACCTGAAAGCTGCTGTCACAATGAAGGCTTTAAATGGTTA  1034
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Query  1035      TAACTGGAAACTGGtttttttttttACATCTTCCGGTTTATATAACATCCCCAACTTTTTG  1094
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Sbjct  16216389  TAACTGGAAACTGTTTTTTTTTTTTACATCTTCTG-TTTATATATCATCTCTA-CTTTTTG  16216446

Query  1095      GAATTTGCTTTATTTTCTCCAATATTGCTAACTTGGAAGATCTTATATATATTATATCAG  1154
                  || |||||||||||||||| |||||||||||||||| |||||||||||||| |||||||
Sbjct  16216447  GA-TTTGCTTTATTTTCTCTAATATTGCTAACTTGGA-GATCTTATATATAT-ATATCAG  16216503

Query  1155      GGGGCAACGTTTCTCTAAGAGGGGATGCCTTTTTATTTCTTTgaaaaag  1203
                  | | ||||||| ||||| || | ||||| |||| ||| | |||| ||||
Sbjct  16216504  GTG-CAACGTT-CTCTA-GATGTGATGC-TTTTAATT-CATTGATAAAG  16216547
```

### A.1.3 *NDC1* knockout PCR product BLAST alignment to At1g73240

*Arabidopsis thaliana* chromosome 1, complete sequence
Sequence ID: ref|NC_003070.9|Length: 30427671
Number of Matches: 1 Range 1: 27543987 to 27544409

Alignment statistics for match

| Score | Expect | Identities | Gaps | Strand |
|---|---|---|---|---|
| 765 bits(414) | 0.0 | 422/425(99%) | 3/425(0%) | Plus/Plus |

Features: hypothetical protein

```
Query  3         AAACAATAGGCATTTATCGTCGAC-ACACATAAAGCACACCAGATAGCTTCAAAGCTTGT  61
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Sbjct  27543987  AAACAATA-GCA-TTATCGTCGACAACACATAAAGCACACCAGATAGCTTCAAAGCTTGT  27544044

Query  62        GTTAGAGCCGAAGGAAGACCAATCTTGAAGCTGAAGAACGGTGGACGCTGTTTTGAAAAA  121
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Sbjct  27544045  GTTAGAGCCGAAGGAAGACCAATCTTGAAGCTGAAGAACGGTGGACGCTGTTTTGAAAAA  27544104

Query  122       ACACAAGTAAGAATCAGCAAAGATACAAAATTGATGAAACATTCCATAAATCAAGAAGCA  181
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  27544105  ACACAAGTAAGAATCAGCAAAGATACAAAATTGATGAAACATTCCATAAATCAAGAAGCA  27544164

Query  182       AAGACACAAAACCTGAATGATAGGAAACTCCAAAACCCATCGTTGCTTAGTAACAAAGAG  241
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  27544165  AAGACACAAAACCTGAATGATAGGAAACTCCAAAACCCATCGTTGCTTAGTAACAAAGAG  27544224

Query  242       GACTGCGTAAAGCAATCCAGTCACTAATCCCCTAAACCCTATTCTTCCAATGGGTCCAAA  301
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  27544225  GACTGCGTAAAGCAATCCAGTCACTAATCCCCTAAACCCTATTCTTCCAATGGGTCCAAA  27544284

Query  302       CGAATCACTAGACAAGCAAAACACAGCAGAGGAGCAGAATCCAGCGAGGGAAGTAGCGCA  361
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  27544285  CGAATCACTAGACAAGCAAAACACAGCAGAGGAGCAGAATCCAGCGAGGGAAGTAGCGCA  27544344

Query  362       GACGAAGAGCACGATGCGTGAAGATACGCGAGCACGGCGGCGGAACTCGGGATCCGAGAG  421
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  27544345  GACGAAGAGCACGATGCGTGAAGATACGCGAGCACGGCGGCGGAACTCGGGATCCGAGAG  27544404

Query  422       GTGAC  426
                 |||||
Sbjct  27544405  GTGAC  27544409
```

### A.1.4 *NDC1* knockout PCR product BLAST alignment to SAIL T-DNA sequence

Sequence ID: lcl|18113Length: 4763Number of Matches: 1
Range 1: 248 to 448
Alignment statistics for match

| Score | Expect | Identities | Gaps | Strand |
|---|---|---|---|---|
| 359 bits(194) | 2e-102 | 199/201(99%) | 2/201(0%) | Plus/Plus |

```
Query  428  CAGGATATATTGTGGTGTAAACAAATTGACGCTTAGACAACTTAATAACACATTGCGGAC  487
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  248  CAGGATATATTGTGGTGTAAACAAATTGACGCTTAGACAACTTAATAACACATTGCGGAC  307

Query  488  GTTTTTAATGTACTGAATTAACGCCGAATTGAATTCGATTTGGTGTATCGAGATTGGTTA  547
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  308  GTTTTTAATGTACTGAATTAACGCCGAATTGAATTCGATTTGGTGTATCGAGATTGGTTA  367

Query  548  TGAAATTCAGATGCTAGTGTAATGTATTGGTAATTTGGGAAGATATAATAGGAAGCAAGG  607
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  368  TGAAATTCAGATGCTAGTGTAATGTATTGGTAATTTGGGAAGATATAATAGGAAGCAAGG  427

Query  608  CTATTTATCC-TT-CTGaaaa  626
            |||||||||| || |||||||
Sbjct  428  CTATTTATCCATTTCTGAAAA  448
```

### A.1.5 *GP210* knockout PCR product BLAST alignment to At5g40480 gene sequence

*Arabidopsis thaliana* chromosome 5, complete sequence
Sequence ID: ref|NC_003076.8|Length: 26975502Number of Matches: 1
Range 1: 16215363 to 16215859
Alignment statistics for match

| Score | Expect | Identities | Gaps | Strand |
|---|---|---|---|---|
| 902 bits(488) | 0.0 | 496/499(99%) | 3/499(0%) | Plus/Plus |

Features: protein embryo defective 3012

```
Query  5         TTTCATCTACTGCTGTTC-TTTGTTTGCATTTAAGTTTCTTCCTTTACCGAGTTATGTCC  63
                 ||||  ||  |||||||||| ||||||||||||||||||||||||||||||||||||||
Sbjct  16215363  TTTC-TC-ACTGCTGTTCATTTGTTTGCATTTAAGTTTCTTCCTTTACCGAGTTATGTCC  16215420

Query  64        AGAGGATTTATTGTAGATGATATTGCATGTATGTTCAACATAATAATTTCCTTTGTGTTG  123
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  16215421  AGAGGATTTATTGTAGATGATATTGCATGTATGTTCAACATAATAATTTCCTTTGTGTTG  16215480

Query  124       ATTGCAGACGGATGACATAAAACTGTATGGAAAAGATTCAGATTATTGGAAAATCGTCTC  183
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  16215481  ATTGCAGACGGATGACATAAAACTGTATGGAAAAGATTCAGATTATTGGAAAATCGTCTC  16215540

Query  184       ACTGCCAGATGAACTTTCCTCTGAATATGGTCAGCGAAATTCTAGAATTTTGAACGCAAT  243
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  16215541  ACTGCCAGATGAACTTTCCTCTGAATATGGTCAGCGAAATTCTAGAATTTTGAACGCAAT  16215600

Query  244       CTCACCAGGATTAGGAGAGCTGACATCTACATTGACTTACTTCAGTGGGCATCAAGAGTC  303
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  16215601  CTCACCAGGATTAGGAGAGCTGACATCTACATTGACTTACTTCAGTGGGCATCAAGAGTC  16215660

Query  304       AAAAGAGGTGAGAAAATTATATATCTTTGGAAAACCTTTTGTCTCTTTCCATTTGTTGGT  363
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  16215661  AAAAGAGGTGAGAAAATTATATATCTTTGGAAAACCTTTTGTCTCTTTCCATTTGTTGGT  16215720

Query  364       ACTGCAAGAGTAACAACTTTGTCTCTAGCAGGTTCTCAAGGTTGTCCAAGAAATTAGGGT  423
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  16215721  ACTGCAAGAGTAACAACTTTGTCTCTAGCAGGTTCTCAAGGTTGTCCAAGAAATTAGGGT  16215780

Query  424       TTGTGAAAAAGTGCAGTTCACATTGAACAGTGAAGATGACACACCTAAGGTTCTACTCCC  483
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  16215781  TTGTGAAAAAGTGCAGTTCACATTGAACAGTGAAGATGACACACCTAAGGTTCTACTCCC  16215840

Query  484       ATGGACCCCTGCTGTTTAT  502
                 ||||||||||||||||||
Sbjct  16215841  ATGGACCCCTGCTGTTTAT  16215859
```

### A.1.6 *GP210* knockout PCR product BLAST alignment to SALK T-DNA sequence

Sequence ID: lcl|43265Length: 12883Number of Matches: 1
Alignment statistics for match #1

| Score | Expect | Identities | Gaps | Strand |
|---|---|---|---|---|
| 390 bits(211) | 2e-111 | 211/211(100%) | 0/211(0%) | Plus/Plus |

Range 1: 6116 to 6326

```
Query  508   CAGGATATATTGTGGTGTAAACAAATTGACGCTTAGACAACTTAATAACACATTGCGGAC  567
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  6116  CAGGATATATTGTGGTGTAAACAAATTGACGCTTAGACAACTTAATAACACATTGCGGAC  6175

Query  568   GTTTTTAATGTACTGGGGTGGTTTTTCTTTTCACCAGTGAGACGGGCAACAGCTGATTGC  627
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  6176  GTTTTTAATGTACTGGGGTGGTTTTTCTTTTCACCAGTGAGACGGGCAACAGCTGATTGC  6235

Query  628   CCTTCACCGCCTGGCCCTGAGAGAGTTGCAGCAAGCGGTCCACGCTGGTTTGCCCCAGCA  687
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  6236  CCTTCACCGCCTGGCCCTGAGAGAGTTGCAGCAAGCGGTCCACGCTGGTTTGCCCCAGCA  6295

Query  688   GGCGAAAATCCTGTTTGATGGTGGTTCCGAA  718
             ||||||||||||||||||||||||||||||||
Sbjct  6296  GGCGAAAATCCTGTTTGATGGTGGTTCCGAA  6326
```

# A.2 PCR product sequences with *Arabidopsis* and T-DNA junctions

### A.2.1 *NDC1* knockout PCR product

Knockout PCR product sequence from *ndc1* homozygous knockout screening. *A. thaliana* genomic junction (light grey shaded) and T-DNA insert junction (dark grey shaded).

```
   1   AAAAACAATAGGCATTTATCGTCGACACACATAAAGCACACCAGATAGCT
  51   TCAAAGCTTGTGTTAGAGCCGAAGGAAGACCAATCTTGAAGCTGAAGAAC
 101   GGTGGACGCTGTTTTGAAAAAACACAAGTAAGAATCAGCAAAGATACAAA
 151   ATTGATGAAACATTCCATAAATCAAGAAGCAAAGACACAAAACCTGAATG
 201   ATAGGAAACTCCAAAACCCATCGTTGCTTAGTAACAAAGAGGACTGCGTA
 251   AAGCAATCCAGTCACTAATCCCCTAAACCCTATTCTTCCAATGGGTCCAA
 301   ACGAATCACTAGACAAGCAAAACACAGCAGAGGAGCAGAATCCAGCGAGG
 351   GAAGTAGCGCAGACGAAGAGCACGATGCGTGAAGATACGCGAGCACGGCG
 401   GCGGAACTCGGGATCCGAGAGGTGACTCAGGATATATTGTGGTGTAAACA
 451   AATTGACGCTTAGACAACTTAATAACACATTGCGGACGTTTTTAATGTAC
 501   TGAATTAACGCCGAATTGAATTCGATTTGGTGTATCGAGATTGGTTATGA
 551   AATTCAGATGCTAGTGTAATGTATTGGTAATTTGGGAAGATATAATAGGA
 601   AGCAAGGCTATTTATCCTTCTGAAAAAAAGGGAAAAACCCG
```

### A.2.2 *GP210* knockout PCR product

Knockout PCR product sequence from *gp210* homozygous knockout screening. *A. thaliana* genomic junction (light grey shaded) and T-DNA insert junction (dark grey shaded).

```
   1   GGGGTTTCATCTACTGCTGTTCTTTGTTTGCATTTAAGTTTCTTCCTTTA
  51   CCGAGTTATGTCCAGAGGATTTATTGTAGATGATATTGCATGTATGTTCA
 101   ACATAATAATTTCCTTTGTGTTGATTGCAGACGGATGACATAAAACTGTA
 151   TGGAAAAGATTCAGATTATTGGAAAATCGTCTCACTGCCAGATGAACTTT
 201   CCTCTGAATATGGTCAGCGAAATTCTAGAATTTTGAACGCAATCTCACCA
 251   GGATTAGGAGAGCTGACATCTACATTGACTTACTTCAGTGGGCATCAAGA
 301   GTCAAAAGAGGTGAGAAAATTATATATCTTTGGAAAACCTTTTGTCTCTT
 351   TCCATTTGTTGGTACTGCAAGAGTAACAACTTTGTCTCTAGCAGGTTCTC
 401   AAGGTTGTCCAAGAAATTAGGGTTTGTGAAAAAGTGCAGTTCACATTGAA
 451   CAGTGAAGATGACACACCTAAGGTTCTACTCCCATGGACCCCTGCTGTTT
 501   ATATTGACAGGATATATTGTGGTGTAAACAAATTGACGCTTAGACAACTT
 551   AATAACACATTGCGGACGTTTTTAATGTACTGGGGTGGTTTTTCTTTTCA
 601   CCAGTGAGACGGGCAACAGCTGATTGCCCTTCACCGCCTGGCCCTGAGAG
 651   AGTTGCAGCAAGCGGTCCACGCTGGTTTGCCCCAGCAGGCGAAAATCCTG
 701   TTTGATGGTGGTTCCGAACGGGGGAAAAAAAAAAAAATATTTTGGAG
```